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Cladosporium cladosporioides: A new report of parasitism on sugarcane woolly aphid, *Ceratovacuna lanigera* Zehntner

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Abstract
Studies were conducted on mycoses of entomopathogenic fungus naturally occurring on sugarcane white woolly aphid, *Ceratovacuna lanigera* Zetner during winter, 2018 at Regional Agricultural Research Station, Anakapalle, India. *Acremonium zeylanicum*, *Beauveria bassiana*, *Cladosporium oxysporum*, *Metarrhizium anisopliae*, *Nomuraea rileyi* and *Verticillium lecanii* were reported to parasitize sugarcane white wooly aphid. During December 2018, a fungus was isolated from parasitized nymphs and adults of *C. lanigera* and characterized as *Cladosporium cladosporioides* based on morphological characters and molecular analysis. The parasitism of *C. cladosporioides* on *C. lanigera* was visualized using light and electron microscopy at the host-parasite interface. Microscopic studies revealed that the nymphs and adults of wooly aphid were completely overgrown by mycelia of *C. cladosporioides*. The mycelium of *C. cladosporioides* has penetrated and disrupted the hydrophobic powdery waxy coating of wooly aphid adult. Overall, our study indicates that *C. cladosporioides* had potential as biological agent for management of sugarcane wooly aphid. This bioagent needs further evaluation under field conditions to establish the potential parasitism of *Cladosporium* isolate against wooly aphid and its effects on other hosts.

Keywords: *Cladosporium*, *Ceratovacuna*, wooly aphid, sugarcane, entomopathogenic fungus

1. Introduction
Sugarcane woolly aphid, *Ceratovacuna lanigera* Zehntner was first reported from West Bengal and subsequently from Northeast India [2]. Severe incidence of *C. lanigera* was noticed for the first time during 2002 in Sangli district of Maharashtra [10]. Later woolly aphid severity was reported from Andhra Pradesh, Bihar, Karnataka Uttar Pradesh, and Uttaranchal. The infestation of sugarcane white woolly aphid has threatened sugarcane cultivation during 2003-2004, particularly in Andhra Pradesh [11]. The nymphs and adults of sugarcane woolly aphid are phloem feeders. They insert their stylets into the host leaves through the natural openings like stomata and obtain their nutrients from the host. Often black sooty growth can be noticed on the honey dew secreted by the insects which reduce the photosynthetic efficiency of the foliage infested with wooly aphid. Further, due to continuous feeding by the nymphs and adults, the crop becomes stunted and leaves dry up from tip backwards. Sugarcane yield and juice quality are affected on continuous feeding of sap [17].
Though effective insecticides are available for management of wooly aphids, spraying of insecticides is not economical in sugarcane ecosystem due to difficulty in spraying, ill effects of chemical pesticides, impermeable nature of rind, etc. Oflate, ecologically sustainable approaches are gaining importance and limited use of insecticides in sugarcane ecosystem has promoted the development of predators like *Dipha aphidivora*, *Micromus* sp. and *Eupeodes confrater* and entomopathogenic fungi like *Acremonium zeylanicum*, *Beauveria bassiana*, *Cladosporium oxysporum*, *Metarrhizium anisopliae*, *Nomuraea rileyi* and *Verticillium lecanii* [9, 15, 16]. In recent years, the use of entomopathogenic fungi has increased, as an alternative to insecticides, due to the great potential they have in pest management and their ecofriendly nature. The present study was aimed at characterization of entomopathogenic fungus naturally occurring on sugarcane wooly aphid and to visualize host colonization through light and electron microscopy.

2. Materials and Methods

2.1 Collection of mycosis sample and isolation of Entomopathogenic fungus (EPF)

During regular monitoring of pests in experimental farm of Regional Agricultural Research Station, Anakapalle ($17^0$40'48" N, $83^0$01'12" E), mycosis was observed in sugarcane white woolly aphid, *Ceratovacuna lanigera*, on sugarcane genotype 2000A 56 during December, 2018. Sugarcane leaves infested with woolly aphids colonized by a brown fungus, were collected from the field in a polythene cover and brought to the laboratory for examination under microscope and further isolation.

The pathogen was isolated from infested wooly aphid colonies on potato dextrose agar (PDA) by standard protocol. Brown mycelia were removed from the nymphs of *Ceratovacuna lanigera* with an inoculation needle and aseptically transferred onto PDA medium. Conidial suspension was prepared in autoclaved double distilled water, after incubation of inoculated Petri plates at 26±1 ^{0}C for 8 days, and transferred to 2% water agar medium. Two days after incubation, tip of mycelium from single germinated conidium was separated with a cork borer and transferred onto PDA medium to obtain pure cultures. The pure culture was transferred onto PDA slants for maintenance and further studies.

2.2 Morphological and molecular characterization

The mycosis of wooly aphid was captured using a stereo binocular microscope (Magnus MSZ-TR). The morphological features of the isolated fungus were determined using compound microscope (NIKON Eclipse E 200) and the images captured digitally using VImage software [13]. Culture (8 days old) from actively growing colonies of the entomopathogenic fungus grown on PDA was used to study the morphological characters of conidia and conidiophores.

Molecular identity of the test fungus was established through homology analysis of internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). The genomic DNA was isolated according to standard protocol [12]. The ITS region of the test fungus was amplified in a PCR using ITS1 and ITS4 primers. The thermocyclic conditions included initial denaturation at 94 ^{0}C for 4 min followed by 38 amplification cycles of 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, 72^0 C for 90 sec followed by final extension at 72 ^{0}C for 10 min. The PCR amplified products were resolved in 1% agarose gel in 1X Tris-acetate EDTA, run for 90 min at 100 V, and the amplified products were excised and outsourced (Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad) for partial sequencing. Similarity of ITS region was aligned using BLAST Programme of GenBank database (NCBI) and phylogenetic tree was constructed using MEGA software version 10.0.

2.3 Ultrastructure of mycosis on wooly aphid

Colonization of entomopathogenic fungus on nymphs of wooly aphid was visualized by scanning electron microscopy. Samples were fixed in 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 hrs at 4^{0}C and post fixed in 1% aqueous osmium tetroxide for 4 h. Subsequently, samples were dehydrated in series of graded alcohols and dessicated to critical point with CPD (EMS 850) unit / vacuum desiccation for 35-45 minutes for complete drying of specimens. The dried samples were seated over the stubs with double-sided carbon conductivity tape, and a thin layer of heavy metal (gold) was coated over the samples by using an automated sputter coater (Model - JEOL JFC-1600) for 180 seconds and scanned under Scanning Electron Microscope (SEM - Model: JOEL-JSM 5600) at required magnifications as per the standard procedures at RUSKA Lab's College of Veterinary Science, PVNR TVU, Rajendranagar, Hyderabad, India [7].

3. Results and Discussion

Wooly aphid incidence was observed from second fortnight of November, 2018 to third week of February, 2019 in the sugarcane genotype, 2000A 56. Maximum infestation of white wooly aphid was noticed during the months of December and January because of low minimum temperatures (5.1-17.2 ^{0}C) and high relative humidity (90-93%). High relative humidity (80-82%) and low sunshine hours (3.2-3.75 h) observed with North East monsoon were found conducive for sugarcane wooly aphid infestation in Srilanka [6]. The pervasiveness of entomopathogenic fungus in the field was found associated with favourable weather conditions prevailing during the period from December, 2018 to February, 2019 {average temperature 23.8 (15.1 to 33.0) and Relative humidity 68% (41% - 93%)}. The increase in wooly aphid population and prevalence of climate conditions and ecological factors favourable for entomopathogenic fungus were found supportive for the mycoses.

3.1 Characterization of entomopathogenic fungus

The fungal colonies were olivaceous in colour with diffuse aerial mycelia with olive black colour on the reverse side. The condidiophores are solitary, brown, unbranched and straight without nodules and produced two types of conidia. Ramoconidia are light brown, cylindrical to oblong in shape and are produced in groups of two to three and measured 4.5–16.8 x 2.1–3.6 µm in size. While, conidia are ovoid to subglobose, catenulate, produced in long chains of upto 8 conidia, aseptate with conspicuous hilum and measured 2.3-4.8 x 1.5-2.6 µm (Fig.1). Based on the morphological characteristics, the fungus was initially identified as *Cladosporium*. As the dimensions of *Cladopsorium* spp. overlap with each other, identification of *Cladosporium* upto species level is a difficult task. However, *Cladosporium* could be identified upto species level through molecular analysis [3].

Fig 1: Morphological characters of *C. cladosporioides* on PDA A) Culture of *C. cladosporioides* on PDA and B) Conidia and conidiophores of *C. cladosporioides*, C) Conidia of *C. cladosporioides*, D) SEM of *C. cladosporioides* on *C. lanigera*

The EPF identified as *Cladosporium* spp. morphologically was further characterized to species level by amplification of ITS region using PCR. The NCBI-BLAST analysis of genomic sequence amplified by ITS1 and ITS 4 has confirmed the EPF as *Cladosporium cladosporioides*. GenBank accession number was obtained on submission of partial gene sequence to NCBI (MN611435). Further phylogenetic tree was constructed to ascertain the grouping of the collected *Cladosporium* isolate (Fig.2). Several sequences from the NCBI database were considered for phylogenetic tree construction. After alignment of the sequences using Clustal W software, phylogenetic tree was constructed using Maximum parsimony method. For the construction of phylogenetic tree MEGA software version 10.0 was used. The results clearly revealed that there is a separate clustering for the different species of *Cladosporium*. The test sequence clustered with *Cladosporium cladosporioides*. The gene sequences of *C. cladosporioides* species were clustered together along with other very closely associated species, *C. pseudocladosporioides*. Similarly, the species *C.oxysporum* has formed a separate cluster. Hence the entomopathogenic fungi naturally occurring on *Ceratolacuna lanigera* was identified as *C. cladosporioides*. Bensh and his coworkers [3] discriminated various species of *Cladosporium* based on molecular phylogeny using ITS (Internal Transcribed Spacer), ACT (Alpha1-anthichymotrypsin) and EF-1α (elongation factor-1) regions.

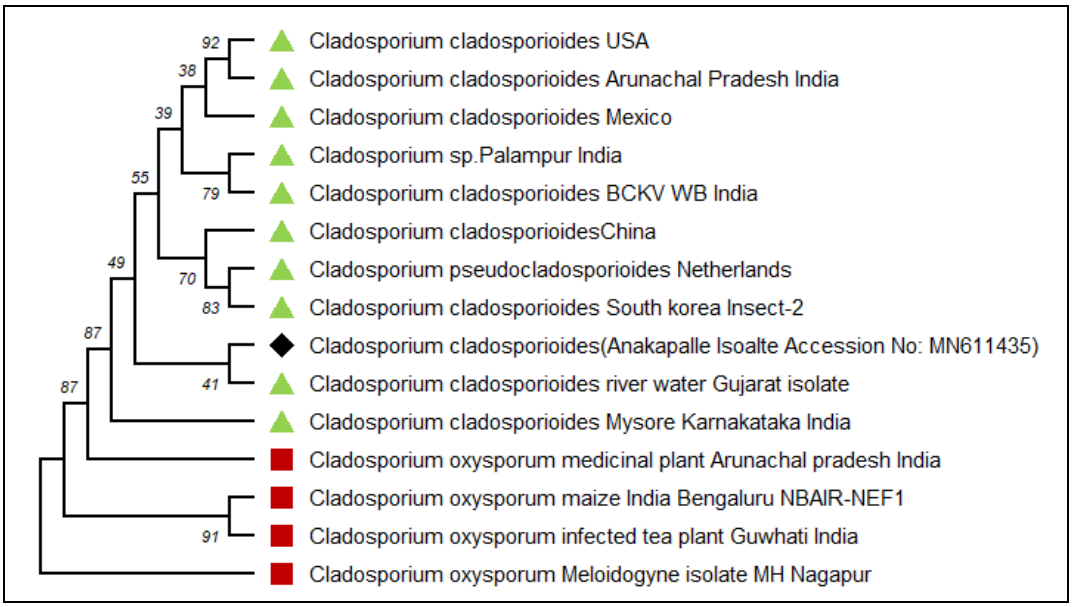

Fig 2: Dendrogram of ITS region sequences of *Cladosporium* using Maximum Parsimony method

3.2 Description of mycosis on wooly aphid

Mycoses on *C. lanigera*, associated with *Cladosporium cladosporioides*, were characterized by the formation of olivaceous green masses on the surface of nymphs and adults (Fig.3).

Fig 3: Parasitism of *C. cladosporioides* on white wooly aphid a) Healthy colonies of wooly aphid on sugarcane variety 2000A 56, b) Parasitized colonies of wooly aphid, c) to-e) Streomicroscopic observation of mycosis of *Cladosporium* on *C. lanigera*

The assemblage of nymphs and adults were found to be superficially colonized by the mycelial growth of the fungus, initially on the powdery wax coating, and subsequently invaded the tissues of the insect. Survival of infected nymphs was observed in the initial stages, but they often did not reach adulthood. The fungus totally invaded the nymphs and adults including the powdery white waxy portions and the mummified bodies of the insect were detached easily from the leaf surface in advanced stages of infection. The ultrastructural study has revealed the colonization of the fungus on the host surface and subsequent disruption of white powdery secretions by the mycelium of *C. cladosporioides* (Fig.4). Then the fungal cells proliferate in the insect body and finally killed the nymphs and adults, and produced numerous conidia under humid conditions.

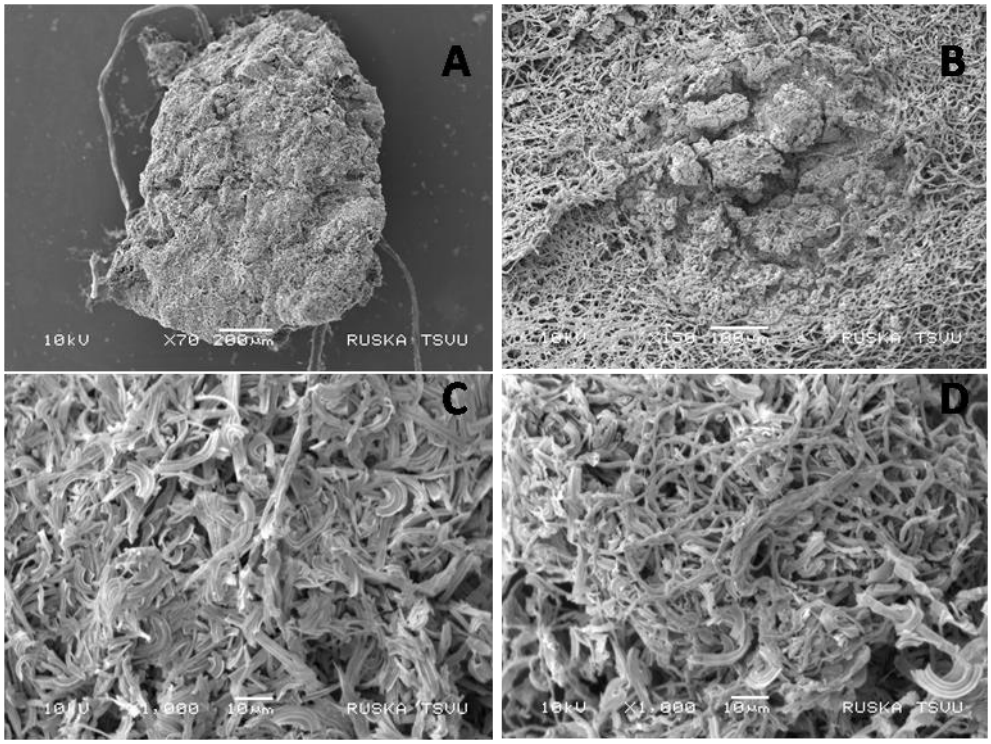

Fig 4: Ultrastructure of mycosis on *C. lanigera* A) Healthy nymph of *C. lanigera*, B) Partially parasitized nymph of *C. lanigera*, C) Powdery wax coating of healthy *C. lanigera* nymph, D) Powdery coating of *C. lanigera* nymph disrupted by mycelial growth of *C. cladosporioides*

The mode of action of entomopathogenic fungi against insects involves attaching to the insect cuticle, followed by germination, penetration of the cuticle and proliferation within the haemocoel, muscles or other tissues of the insect body to collapse the host immune system [8]. *C. cladosporioides* also elaborates secondary metabolites, 3-phenyl propanoic acid and 3-(4β-hydroxy-6-pyranonyl)-5isopropylpyrolidin-2-one, which are toxic to the nymphs and adults of cotton aphid [14].

The efficacy of *Cladosporium* spp. as an entomopathogenic fungus was reported against whiteflies and aphids in Egypt. *Cladosporium uredinicola* was reported as the predominant species naturally infecting whiteflies and aphids [1]. Natural infection by *Cladosporium* ranged from 18.19 to 44.38 per cent, especially on whitefly nymphs. Similarly, our results have shown rapid colonization of nymphs by *C. cladosporioides* than the adults. The ecological factors like economic injury level of the pest, ecosystem and environment, timing and transmission of pest and host resistance were attributed as major factors contributing to the success of entomopathogens in insect control [5]. Further, factors like host range, specificity of EPF, availability of nutrients, physiological state of the host, physical barriers of the pest, viz., cuticle and epi-cuticular structures, and defense mechanisms were reported to influence colonization of insects by entomopathogenic fungi [4]. In the present study, the mycosis of *C. cladosporioides* on *C. lanigera* was also found to be affected by the population of white wooly aphid, climatic factors operative from December to February and prevalence of pathogen inoculum. The existence of high humidity in sugarcane crop canpy is a favourable habitat for rapid multiplication and dispersal of entomopathogenic fungi and thus is an ideal ecosystem for successful management of sugarcane pest by biological means.

4. Conclusions

Various entomopathogenic fungi, *viz*., *Metarhizium anisopliae, Verticillium lecanii, Beauveria bassiana* and *Nomuraea rileyi*, were tested against white wooly aphid, *Ceratovacuna lanigera* under laboratory and field conditions. Though these fungi were found efficient under laboratory conditions, their potential was not completely harnessed under field conditions. In the present study, *C. cladosporioides* Anakapalli isolate reduced the incidence of white woolly aphids, and hence might be utilized as one of the components of integrated pest management module in sugarcane. Further the isolate has to be tested for its antagonism against wooly aphid at different concentrations on nymphs and adults of *C. lanigera*, survival at various temperatures and humidity levels, time to colonize its host (incubation period) under controlled and field conditions, multiplication and survival in different substrates, effect on other organisms, etc., prior to commercial utilization. The present investigation provides a basis for further research on the utilization of *C. cladosporioides* as a potential biocontrol agent against sugarcane white wooly aphid.

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