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New Report of Pre-emergent Inflorescence Rot of Coconut from India

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ABSTRACT: Coconut inflorescence sap tapped from the unopened spathe or inflorescence of coconut commonly called as neera in Karnataka is a healthy and therapeutic drink which is rich in Vitamin-C and one of the natural product derived from coconut. During pre winter period in and around the coconut growing areas of Bhadravathi taluk of Shivamogga district many coconut palms were found expressing the inflorescence rot symptoms which was not yet recorded earlier in neera extracting coconut palms. The affected spathe appeared to be yellowish green, spathe when made a fine cut at around 45º angle for neera collection. It was observed that the pathogen enters through the cut end and initially moves through the individual rachillae and as the disease progresses it infected all the floral organs and turns them into brown to blackish color resulting in rotting of entire inflorescence. Such inflorescence emitted foul smell. From such inflorescence a fungus was isolated and identified as *Thielaviopsis paradoxa* through cultural, morphological and molecular methods.

Keywords: Pre-emergent inflorescence rot, *Thielaviopsis paradoxa*, Characterization, Coconut.

INTRODUCTION

Coconut (*Cocus nucifera* L.) is a widely grown plantation crop of India covering a wide area across tropical and subtropical regions. It is often referred as tree of life, as it provides food, nutrition and livelihood to millions of coconut farmers in Asia and Pacific region (Chan, E. and Elevitch). Indonesia, Philippines and India are the largest producers of coconut in the world. In India, annually production is 23,904 million nuts covering an area of 20.8 lakh hectare with a productivity of 11,481 coconuts/per hectare (Anon., 2020). 90 per cent of the total production from the country is from Four southern states *viz*., Kerala, Tamil Nadu, Karnataka and Andhra Pradesh. For decades coconut products have held a respected and valuable place in Indian culture. In India, the use of coconut for food, and its applications in the Ayurvedic medicine were documented in Sanskrit 4,000 years ago. It is proven to be antiblenorrhagic, antibronchitis, febrifugal, and antigingivitic (DebMandal and Mandal, 2011). A variety of products were being derived from the coconut palm *viz*., edible kernel, oil, shell, tender coconut water, fiber, sap from inflorescence, sugar, fuel, and the industrial uses include soaps, detergents and bio-diesel. Among the products from coconut, inflorescence sap is the most remunerative product for farmers. Coconut inflorescence sap tapped from the unopened spathe or inflorescence of the coconut, is one of the versatile food products derived from coconut. It can be used as a nutrient-rich health drink and also be processed to syrup, honey and coconut sugar (Van Die, 1974). It is a natural sugar containing health drink and a rich source of minerals, amino acids and vitamins (Hebbar *et al*., 2015). Globally, neera and its value-added products are produced and marketed on a commercial scale. It is widely consumed in India, Sri Lanka, Africa, Malaysia, Indonesia, Thailand, and Myanmar. It contains ascorbic acid, nicotinic acid and riboflavin (Jayashree, 2013). Besides, it plays an important role in the Indian economy, contributing $105 million per year to the national GDP (Ghosh *et al*., 2018). But in the recent days due to the climate change the severe rotting in the unopened spathe was observed in Baranduru village of Bhadravathi taluk of Shivamogga district in severe form. So the present investigation was taken up to isolate, identify and prove the pathogenicity of the causal agent.

MATERIALS AND METHODS

A. Collection of infected sample and isolation of the fungus

Pre emergent spathe showing typical rotting symptoms were collected from the field and subjected to isolation

following standard tissue isolation method. These tissues were surface sterilized with 0.1 per cent sodium hypochlorite solution for two min followed by thorough rinsing in sterilized distilled water. The tissues were placed on potato dextrose agar medium under aseptic conditions and incubated at 27°C and observed for the fungal growth. Upon appearance of fungal growth on the media, a loop of fungal culture from the colonies was picked and placed on a clean glass slide containing a drop of lacto phenol dye. Isolated fungus was identified based on the cultural and phylogenetic characterization.

B. Proving Pathogenicity

Pathogenicity test was conducted both *in-vitro* and *in-vivo*. From the pure culture conidial suspensions was prepared using sterile distilled water. For *in-vitro* studies two unopened healthy spathe were detected and selected from a healthy coconut plant and the upper external surfaces was surface-sterilized with 95 per cent ethanol. The flowers and strands of detached spathe were inoculated by injecting 1 ml of conidial suspension (10×10^{-5} CFU) at three spots maintaining 10 cm distance between two spots on the inflorescence. Whereas, sterile distilled water (1ml each) at three places were inoculated and utilized as check (control) and Inoculated spathes were incubated in polythene covers at 25°C. After seven days of inoculation, spathes were examined for infection and upon observing similar symptoms, the pathogen was re-isolated, purified and identified whereas, similar procedure was followed to prove the pathogenicity directly on plants (*in-vivo*) and similar results were obtained, thus proving Koch's postulates.

Injury made for inoculation | Inoculating the Mycelial bit | Inoculating with Sterile distil water

Spathe covered with polythene | Symptom Appearance | Pure culture of *Thielaviopsis*

Plate 1. Proving Koch's Postulate in the laboratory.

C. Genomic DNA extraction

Pure culture obtained from infected inflorescence was used for genomic DNA extraction. The pathogen was grown on PDA medium for twelve days at 25°C. The obtained mycelium was scraped from fully grown pure culture plate using a sterile scalpel and it was added to a sterilized pestle and mortar. The DNA was extracted by employing modified cetyl trimethyl ammonium bromide (CTAB) protocol (Moller *et al*., 1992).

D. PCR amplification

The ITS/5.8S regions were amplified using primer pair ITS1 (5' -TCCGTAGGTGAACCTGCGG-3') hybridizes at the end of 18S recombinant deoxyribonucleic acid (rDNA) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and at the beginning of 28S rDNA (Ferrer *et al*., 2001). Amplification was carried out in 25 µL reaction mixtures containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.6 mM of each dNTPs, 0.25 µM of each primer, 1.25 U *Taq* polymerase (Promega) and 4 ng gDNA. PCR was performed in a thermal cycler. PCR cycling profile: hot start at 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were resolved on 1% (w/v) agarose gel (with ethidium bromide, 10 ng/100 ml).

E. Sequencing and in silico analysis

The ITS region was sequenced for the obtained isolate. The PCR product was sequenced using forward and reverse primers at Biokart, India. Bengaluru. Homology search was done using BLAST algorithm available at the www.ncbi.nlm.nih.gov.

Healthy inflorescence Infected inflorescence after inoculation

Infected inflorescence after 20 days after inoculation

Plate 2. Proving pathogenicity under Natural Field conditions

RESULTS AND DISCUSSION

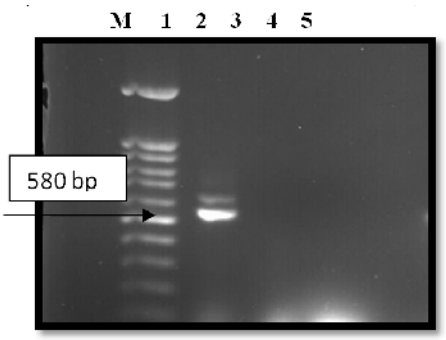
The inflorescence rot symptoms were obvious at the emerging spathes during winter. It mainly appeared on the external surface of unopened spathes which bared rusty lesions. Similar lesions were also seen inside the spathes and on the flowers which later turned into a brownish color. The symptoms appeared mostly near the top of spathe and the disease invasion resulted in complete destruction of flowers. However, some of the infected spathes remained unopened and dried. The pathogenicity was proved both under *in vitro* as well as *in vivo* under field conditions. Under lab conditions, the healthy spadix was artificially injured using heat sterilized and cooled needle in at three spots maintaining 10 cm distance between two spots on the inflorescence. Whereas, sterile distilled water (1ml each) at three places were inoculated and utilized as check (control) by employing mycelial bit inoculation method and Inoculated spathes were incubated in polythene covers at 25°C. The inoculated spadix was wrapped using a polythene cover to artificially create the humidity under lab conditions. Also under field conditions the pathogen was artificially inoculated to the healthy coconut spadix by artificially damaging the spadix in 3 different places and the inoculated spathe was covered with the polythene cover. Observations were recorded for the development of typical rotting symptoms. The symptoms started appearing after seven days of inoculation (*in-vitro*) and twelve days of inoculation under field conditions. The pathogen was re-isolated from the infected spadix and the symptoms and the colony of the pathogen were compared with the original to confirm identity and pathogenicity of pathogen.

Under microscope (400X) the mycelium of the fungus appeared pale brownish with straight conidiophores up to 250 µm in length. Each had a terminal phialide. The conidia were cylindrical colorless to pale brown. Based on the mycelium and conidia produced in the culture the fungus was initially identified as *Thielaviopsis paradoxa*. The obtained results were in accordance with the results obtained by Dulce and Edson, (2009).

A. Amplification of ITS1 and ITS4 region

Genomic DNA of the fungus observed by electrophoresis in 1.2 per cent Agarose gel was about 7-8 Kb with approximate concentration of 91ηg μg^{-1}. PCR amplification was observed with the amplicon size of 580 bp with a concentration of around 150 ηg μg^{-1}.

This is the first report of amplification of ITS rDNA region of *Thielaviopsis paradoxa.* Since this is the first report of inflorescence rot in coconut, the sequence of ITS rDNA region of was deposited in the Genbank and the accession number MW 599825 was obtained. PCR-based method is considered as a fast and more sensitive technique compared to the morphological identification. The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal repeat unit has become the primary genetic marker for molecular identification and other species-level pursuits in many groups of fungi (Seifert, 2009). The identification of fungi at species level has been based mostly on the use of variable ribosomal-DNA (rDNA) ITS regions.



M: 100 bp Ladder, 1= Infected Coconut sample
Plate 3. ITSrDNA amplication of *Thielaviopsis paradoxa.*

CONCLUSION

Neera is a unfermented sap tapped from the immature inflorescence of coconut. As it contains sugars, minerals and vitamins it is considered to be one of the best natural health drink. Although there is no history of any plant pathogen affecting the neera production tilldate many coconut palms were found expressing the pre emergent inflorescence rotting like symptoms during pre winter period in and around the coconut growing areas of Bhadravathi taluk of Shivamogga district. Therefore from the present study it was revealed that *Thielaviopsis paradoxa* to be the exact cause for the pre emergent inflorescence rotting in coconut. To the best of our knowledge this the first report of *Thielaviopsis paradoxa* causing inflorescence rot of coconut in India. Hence the sequence of ITS rDNA region of was deposited in the Genbank. However in the future days the multi-locus phylogenetic analyses of the pathogen will be studied in detail.

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Conflict of Interest. None.

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