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First report on molecular characterization of Indian canna rust caused by *Puccinia cannacearum*

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Abstract
Canna (*Canna indica* L.) a popular landscape ornamental and hardy plant prone to be affected by few fungal and viral diseases. However, in Karnataka the rust disease of canna caused by *Puccinia cannacearum* is noticed earliest at College of Horticulture, Bengaluru, Karnataka during the year 2016-17 and this is first attempt to characterize this rust pathogen from India. Symptoms of canna rust include: Initially, numerous orange-yellowish rusty spots on the lower surface of the leaves and on leaf sheath which turns brown and later covers the entire leaf area with coalescing pustules, eventually leads to premature senescence and severely infected leaves wither away. Microscopic examination of these rust pustules revealed the presence of golden pale yellow colored egg to pear-shaped uredospore (28-55 x 20-35 µm) with bristly walls and cylindrical to club-shaped teliospores (50-83 x 14-21 µm). Further, the pathogen was amplified and confirmed using ITS-rDNA primers with an amplicon size of 700 bp and phylogenetic analysis of the data revealed that the Indian isolate used under the study was found to be matched with China isolate (Accession no. JQ303103.1) of about 80% identity and the Indian isolate sequence was deposited in Gen Bank (Accession no.MH654794).

Keywords: Canna, fungal disease, *Puccinia cannacearum*, Gen Bank, sequencing

Introduction
Canna (*Canna indica* L.) a perennial herb and popular landscape ornamental species commonly called as canna lily or Indian canna (Family: Cannaceae). It is native to South and Central America including the Caribbean and grows well in various tropical and subtropical climates. It produces broad, flat, alternate leaves with solid green but some cultivars have brownish or even variegated leaves and large-sized colorful garden flowers which can also be used in herbaceous borders and as patio/decking plant (Chopra *et al*. 2013) [3]. It has many medicinal uses and fumigated stem and leaves are used as an insecticide and the plant can be used for waste water treatment.

Generally, *Canna indica* is a hardy plant with a few fungal diseases caused by *Fusarium*, *Rhizoctonia*, Botrytis blight, leaf spot-*Cercospora cannae* (Kar & Ray, 1985) [5] and rust-*Puccinia cannacearum* from Andhra Pradesh, India (Bagyanarayana & Ramesh, 1999) [1] indicated only as a new rust taxon, but characterization was not done so far and viral diseases like *Canna mosaic virus*, *Canna mottle virus* and *Canna yellow mottle virus*. However, in Karnataka, the rust disease of *Canna indica* caused by *Puccinia cannacearum* is noticed earliest at College of Horticulture, Bengaluru, Karnataka during the year 2016-17 and is recurring year after year regularly in a severe form. This rust fungus is an obligate biotroph that belongs to the Kingdom: Fungi, Phylum: Basidiomycota (Subphylum: Pucciniomycotina) Class: Pucciniomycetes and Order: Uredinales. ITS regions were highly variable between taxonomically distinct fungal species (De Backer, 2012; Gardes *et al.*, 1991) [4]. Hence, they were the good target for phylogenetic analysis (Bruns *et al.*, 1991; Larena *et al.*, 1999; Sugimura, 2001; White *et al.*, 1990) [2, 7, 9, 10]. Kropp *et al.* (1997) [6] rDNA ITS universal primers were successful for the amplification of dyer's wood rust fungus and other species of *Puccinia* from crucifers.

This rust pathogen causes severe quality loss and affects the beauty of environment. The information available on this rust pathogen was very much less. Hence, the characterization of *Puccinia cannacearum* was set out for the investigation.

Material and Methods
Morphological characterization
Canna indica plants showing the typical symptoms of rust were labeled in the field from the first day of symptom expression and infection on different parts of the plant were observed and

recorded. Then, the diseased samples were sectioned and observed under microscope and image was photographed using microscopic camera Nikon D-7000 with the microscopic adopter. Parameters measured were length and breadth of uredospores and teliospores using stage micrometry. Colour, shape and type of spores were observed by visual observation.

Molecular characterization

The rust infected canna leaves showing the typical symptoms of rust of *Canna indica* was collected in a paper bag. Further, presence of uredospores and teliospores was confirmed by microscopic examination before the isolation of genomic DNA. Total genomic DNA was isolated using Cetyl-Trimethyl Ammonium Bromide (CTAB) method (Pedley, 2009) [8] with slight modification with the addition of Proteinase K and a pinch of celite, in order to get good quality DNA and quantified by using nano-drop. The quality of DNA extracted was tested using 1 per cent agarose gel electrophoresis.

PCR amplification

The template DNA was amplified using universal ITS-rDNA primers ITS4 (5'-TCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). Amplification was done in PCR tubes with a total reaction mixture of 25 µl containing the following components: PCR ready mix buffer (1X) – (8.5 µl), forward primer (ITS-5)10 pM/ µl (2.0 µl), reverse primer (ITS-4)10 pM/ µl (2.0 µl), 5 ng template DNA (2.0 µl) and Sterile water (10.5 µl). PCR reaction was carried out using Eppendorf Master cycler and PCR was programmed for 35 cycles as follows: Initial denaturation 5 min @ 94 ºC, denaturation 30s @ 94 ºC, annealing 30s @ 48 ºC, primer extension 60 s @ 72 ºC and final extension 8 min @ 72 ºC. The amplified PCR products were sent for gene sequencing. The sequence so obtained was used for NCBI-blast analysis using Bioedit, ClustalW and phylogenetic relationship of *Puccinia cannacearum* was obtained using MegaX.

Results

Morphological characterization

Initially, numerous orange-yellowish rusty spots on lower surface of the leaves and on leaf sheath was observed (Figure 1) later these rusty pustules turns brown and later covers the entire leaf area with coalescing pustules, eventually leads to premature senescence and severely infected leaves falls off. Under 40X magnification of microscopic examination, the rust pustules reveals the presence of golden pale yellow coloured uredospores with bristly walls which vary from egg to pear shaped measuring 28-55 x 20-35 µm (Figure 2). With the progress of the disease, dark brown rusty pustules had teliospores (Figure 3) which are cylindrical to club shaped ranged from 50-83 x 14-21 µm with thickened acute apices and short pedicel was seen. No pycnial and aecial stages were observed.

Molecular characterization

Further, the pathogen was confirmed using ITS-rDNA primers ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The amplified product of 700 bp (Fig. 4) was sequenced and aligned using ClustalW in Bioedit program and phylogenetic analysis of the data revealed that the Indian isolate under the study was found to be matched with China isolate (Accession no. JQ303103.1) of *Puccinia cannacearum* from *Canna indica* with 80% identity (Fig. 5) and the sequence has been deposited in GenBank (Accession no.MH654794).

Discussion

This is the first detailed report on morphological and molecular characterization of rust of *Canna indica* caused by *Puccinia cannacearum* in India. Orange-yellowish rusty spots on lower surface of the leaves and on leaf sheath later these orange-yellowish rusty spots turns brown and later covers the entire leaf area with coalescing pustules, eventually leads to premature senescence and severely infected leaves wither away. The presence of uredospores (secondary inoculum) and teliospores (resting stage) indicate the signs of rust disease. No pycnial and aecial stages were observed and still unknown.

For isolation of DNA, the protocol was modified slightly (Pedley, 2009) [8]. Where, with the addition of Proteinase K (0.3 mg/ ml) and a pinch of celite yielded a good quantity and quality DNA. Proteinase K which removes proteins and celite acted as abrasive and disrupted the fungal cell wall, that extracts the DNA easily into extracting solution. The good quality and purified DNA was used as a template for PCR assay. Universal ITS-rDNA primers were used as explained in material and methods for amplification and pathogen was confirmed with ITS-4 and ITS-5 primers corresponding to the region of 5.8S rDNA gene with an amplified product of 700 bp (Figure 4). The amplified PCR products were sequenced and phylogenetic relationship of *Puccinia cannacearum* with other rust species are obtained using neighbour joining and bootstrap method with 1000 replications in MegaX Software (Fig 6). Only one cluster was formed with respect to fungal hierarchy. The outgroup *Pythium* was far and the separate clusters were formed with Order: Uredinales and differ at genus and species level. *Puccinia cannacearum* fell apart from other rust species *viz.*, *Coleosporium*, *Puccinia* sp. and *Uromyes* but it was closely associated and showed 80% homology (Figure 5) with the *Puccinia cannacearum* of China isolate (Accession no. JQ303103.1). This confirmed the presence of pathogen that belongs to rust species using universal eukaryotic ITS regions of fungi (Bruns *et al.*, 1991; White *et al.*, 1990) [2, 10] and the sequence has been deposited in GenBank (Accession no.MH654794).

To our knowledge, this is the first fully described record and most important fungal disease in *Canna indica*. Hence there is a need for further investigation on occurrence and severity of disease, all spore stages, survival and spread mechanism of this pathogen and integrated disease management strategies to prevent the entry of this rust pathogen into unaffected areas.

Fig 1a, b: Orange-yellowish rusty pustules on lower surface of the leaf and on leaf sheath

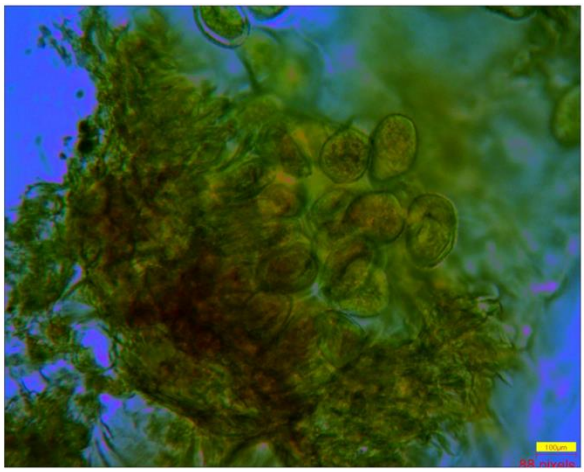

Fig 2: Golden pale yellow coloured egg to pear-shaped uredospores with bristly walls

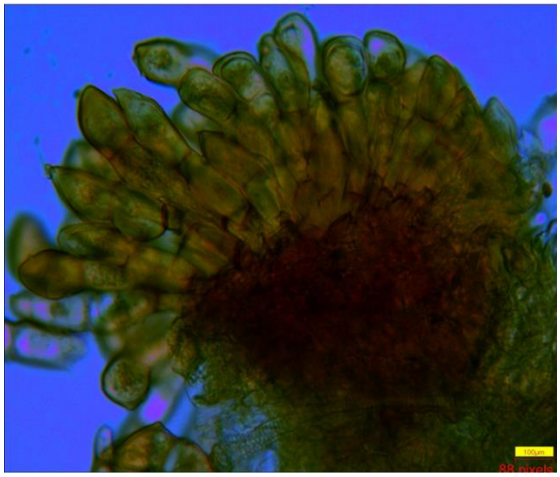

Fig 3: Microscopic view of club-shaped teliospores in telium

Fig 4: The PCR amplified product of 700 bp with ITS 4 and 5 primers

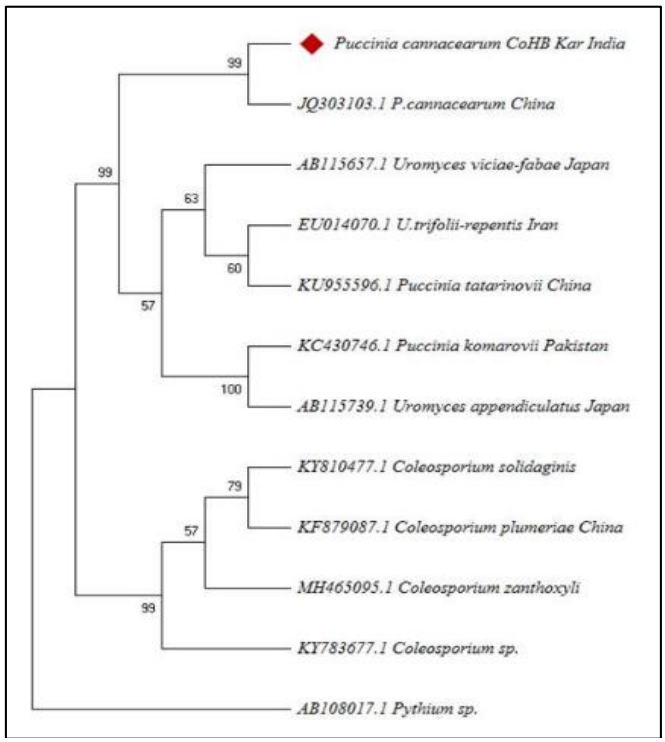

Fig 5: Dendrogram showing the phylogenetic relationship of the *P. cannacearum* with other Gen Bank accessions

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