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Isolation, characterization and reaction study of *Pestalotia palmarum* inciting grey leaf blight on coconut

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Abstract
The grey leaf blight disease (GLBD) like symptoms was observed in severe form on the farm of the NAU on the *Cocos nucifera*. The diseases samples were collected and isolated on PDA medium, by morphological characterization and using standard references to identification of fungi and also by sending fungus culture to IARI, New Delhi, pathogen species confirm as *Pestalotia palmarum* (Cooke) Stey. Pathogenicity assay and Kochs fulfilment was proved that the pathogen to be constantly associated with the disease. The current investigation was under taken to study the actual cause of the symptoms observed on the plant and their identification, pathogenicity as well as the host range of the identified pathogen to manage the further cause.

Keywords: *Cocus nucifera*, leaf spot disease., identification, Koch's postulate, host investigations

1. Introduction
The coconut tree (*Cocos nucifera* L.) is a native of Malaysia or Indonesia and is well known as a 'King of Palms'. (Rethinam, 2003) [12]. The significance of the palm tree is found in the fact that it not just offers people with shelter, water, and food, but also serves as the basis for a number of businesses that are closely related to both home and commercial life. In India, coconut acts an crucial role in the social, economic and cultural activities of the people. The coconut plant (*Cocos nucifera* L.), the sole species found in the genus Cocos, is a big palm that may grow to a height of 30 metres. It has pinnate leaves that are 4-6 metres long and pinnae that are 60-90 cm long. Old leaves neatly fall off the tree when they become old. Seed propagation is the commercial propagation in coconut. The palm continues to produce for more than 80 years, and it takes 10 to 15 years after planting to reach its peak bearing potential (Karthikeyan and Bhaskaran, 1999) [6]. Coconut is predominantly cultivated in southern states of India viz. Kerala, Tamil Nadu, Karnataka and Andhra Pradesh. In India, Kerala ranks 1st in production and area of coconut while, Tamil Nadu ranks 2nd in production. In India, coconut is grown in an area of 1.94 million hectares with an annual production of 12,833 million nuts and a productivity of 6632 nuts/ha. (Samiyappan *et al*., 2006) [13]. In Gujarat state, Coconut covers an area of 15,170 hectares with a production of 135366000 nuts. The 100 gm of edible portion of coconut contains protein, moisture, fat, minerals, fibre, carbohydrates, calcium, phosphorus, iron *viz*., 4.5%, 36.3%, 41.6%, 1.0%, 3.6%, 13.0%, 10 mg, 240 mg, 1.7 mg respectively and vitamin C, vitamin B complex 1 mg. (Chauhan *et al*., 2008) [1]

Many fungal diseases have been observed on coconut at various stages of growth and production. Among the fungal diseases, grey leaf blight [*Pestalotia palmarum* (Cooke) Stey.], Bud rot [*Phytopthora palmivora* Butl.], Stem bleeding [*Ceratocystis paradoxa* (Dade) Moreau], Wilt [*Ganoderma lucidum* (Leyss) Karst.], Leaf rot [*Bipolaris halodes*, *Gloeosporium* sp., and *Gliocladium roseum*], Anthracnose [*Glomerella cingulata* (Stonem.) Spaulding & Schrenk], Leaf spots [*Botryosphaeria disrupta* (Berk. & M.A. Curtis) Arx & E. Muller, *Curvularia lunata* (Wakk.) Boedijn, *Drechslera halodes* (Drechsler) Subram. & Jain, *Drechslera gigantea* (Heald & Wolf) Ito]. This GLBS of coconut is now of common occurrence in all the coconut growing areas of the world. Both nursery seedlings and mature palm trees suffer serious harm as a result of the disease. The occurrence of leaf blight caused a 10–24% decline in coconut output (Karthikeyan and Bhaskaran, 1998). [6] The disease causes irregular greyish lesions on the leaflets. In severe cases, several lesions coalesce together to form irregular necrotic patches resulting in defoliation of leaves (Das and Mahanta, 1985) [2]. The GLBD of coconut caused by *Pestalotia palmarum* (Cooke) Stey., was also reported around the coastal regions of Orrisa (Das *et al*., 1985) [3].

The GLBD was observed in severe form on the farm of the Navsari Agricultural University, in the year 2007 on the *Cocos nucifera* and *Pestalotia palmarum* (Cooke) Stey. was observed to be constantly associated with the disease. The current experiment was under taken to study the actual cause of the symptoms observed on the plant and their identification, pathogenicity as well as the host range of the identified pathogen to manage the further cause.

2. Material and Methods

2.1 Sample collection and isolation

Samples of coconut GLB with typical symptoms were obtained from the college farm at NAU in Navsari and transferred to the lab where they were air dried, cleaned with clean water, placed onto blotting papers under a herbarium press, and preserved for later research. The isolated causative agent was determined by microscopic inspection of the collected samples. The standard isolation technique was used to carry out the isolation, subculture and maintained of the culture (Khaire *et al*., 2023; Kamthe *et al*., 2023) [7, 5]

2.2 Pathogenicity test

Pure sporulating culture of the fungus isolated from infected leaves was used for proving the pathogenicity. Spore suspensions (95 conidia/450X magnification field) from 8 days old culture was prepared by through homogenization of mycelial mass in sterilized distilled water followed by filtration through muslin cloth to remove mycelial bits. This was then inoculated on the leaves. The pots were watered up to saturation in the morning and the inoculation was carried out in the evening hours. The leaves to be inoculated were washed with distilled sterilized water before inoculation. The inoculation was carried out on leaves by following three methods:

1. Pin pricking: Sterilized pins were used to prick the leaves at 3 to 4 place and inoculum was applied with sterilized cotton swab.
2. Tooth brush injury method: Sterilized tooth brush was dipped in the suspension and inoculated with gentle rubbing on leaves at 3 to 4 places.
3. Spraying of spore suspension (without injury on leaves)

Application of mycelial and suspended spores of *Pestalotia* sp., which was isolated from coconut leaves, was used to inoculate the pathogen on leaves. Only distilled, sterilised water spray was used for appropriate controls. Then, to keep high humidity (90 to 100%) surrounding the plants, all of the plants were maintained within wet polythene bags that were often sprayed with distilled, sterilised water. The observations regarding infection and the emergence of symptoms were noted. The fungus was once again isolated from diseased leaves that had been infected and were showing signs of GLB, and its morphological and cultural characteristics were compared to those of *Pestalotia* sp., which had previously been identified from sick coconut leaves.

2.3 Pathogen identification

The pathogen inducing GLB of coconut was identified by examining its morphological and cultural characteristics. The cultural traits were noted from the beginning of growing through the first 15 days. Using a stage and an ocular micrometre, the length and diameter of spores from a culture of *Pestalotia* spp. that had been growing for 10 days were taken under low power magnification and compared to those described in the literature. The hyphae, spores, and acervulus have been microphotographed. For additional confirmation and identification, the pure culture was also sent to the Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology (DMPP), I.A.R.I., New Delhi- 110 012.

2.4 Host Range

To study the host range different host viz., mango, sapota, guava and ber were screened for their susceptibility to the coconut grey leaf blight pathogen *P. palmarum*. The detached twigs with leaves of mango, sapota, guava to be screened and detached fruits of guava and ber were inoculated by pin pricking method and using spore suspension containing 1.6 × 106 spores/ml of *P. palmarum*. Inoculated twigs and fruits were observed periodically at 24 hr for development of grey blight symptoms.

3. Results and Discussion

3.1 Collection and isolation and Symptomatology of test pathogen

The diseased coconut leaves with the usual GLB symptoms were obtained from the NAU farm in Navsari and were subjected to tissue isolation, which produced a pure culture of *Pestalotia* spp. The culture was maintained on PDA slants for future research after being further purified through periodic subculturing. During a visit to the horticulture farm, the afflicted plants were seen, and the symptoms of the disease on coconut are detailed below. The GLB first showed up on the leaflets as tiny yellow specks enclosed by a greyish band in the field (Fig. 1A). Later, these spots combine to form erratic necrotic patches. The middle gradually becomes whiter than brown, and the brown band darkens (Fig. 1B). In cases of severe illness, the leaf blade completely shrivels and dries out (Fig. 1C). Das and Mahanta (1985) [2] said that *Pestalotia palmarum* (Cooke) Steyaert on coconut caused irregular greyish lesions on the leaflets. In severe cases, several lesions coalesced together to form irregular necrotic patches. Joshi and Raut (1992) [4] reported *Pestalotia versicolor* (Speg.) Steyaert causing grey leaf blight of clove and observed that initially, brown pin-head like spots appeared on the upper surface of leaves. These brown spots were always surrounded with yellow halo. Later on, these spots turned white-grey and enlarged in size, coalesced and resulted in blightening of the leaves.

Fig 1: Symptomatology of GLB pathogen caused by *Pestalotia sp.* on coconut tree (A) Field view of diseased coconut tree with GLB caused by *P. palmarum* (B) Extensive leaf blightening caused by *P. palmarum* C) Close up view of GLB showing typical ashy white necrotic areas (D) Pure culture of *P. Palmarum* isolated from infected leaves of coconut.

3.2 Identification and morphological characteristics of the pathogen

Pestalotia spp. isolate was isolated by tissue separation from coconut infected leaf lesions (Fig. 1D). The isolate produced on PDA's physical and cultural traits were investigated and contrasted with those stated in the literature. *Pestalotia palmarum* (Cooke) Steyaert (ITCC. No.-6898.08) was identified as the pure culture when it was delivered to the ITCC, DMPP, IARI., New Delhi-110 012. Additionally, the fungus manifested GLB symptoms during a pathogenicity test. Thus, *Pestalotia* spp. under study was identified and confirmed as *Pestalotia palmarum* (Cooke) Steyaert. Colony grew rapidly on PDA medium and covered the whole 90mm diameter Petri plate within 8 days at room temperature (minimum 27.84 °C and maximum 33.93 °C). The mycelium was initially hyaline when young and later turned white in colour (Fig 1D). In all cases *P. palmarum* was cottony white with black acervuli (Fig 1D), mycelium fluffy white, hyphae hyaline, septate, branched, 2.2-3.3 µm wide (Fig 1D); acervuli small, black, circular, scattered or sometimes more or less aggregate; conidia five-celled, fusoid, 20-27.5 × 7.5-10 µm (average 23.125 × 8.214 µm), coloured cells 3, cinnamon brown, constricted near septa 20-27.5 µm long, end cell hyaline [Fig 2 ABCD.], upper cell bearing 3 spathulate, hyaline setulae, 19.5 µm long [Fig 2D], basal cell conoid with pedicel 7.5 µm long. These morphological and cultural characters of isolated *Pestalotia* spp showed its close identity with *Pestalotia palmarum* (Cooke) Steyaert. described by Rai *et al*. (1982) [11].

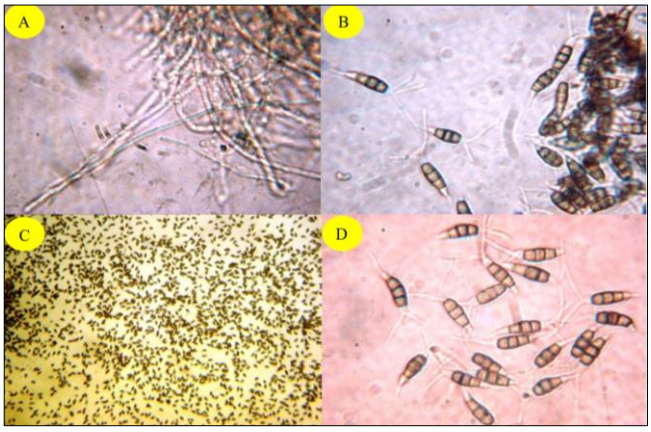

Fig 2: Morphological characteristics of *Pestalotia* sp. (A) Microphotograph of colourless septate hyaline mycelium of *P. palmarum* at high power magnification, 450 X (B) Conidia of *P. palmarum* with three hyaline setulae of front end of spore at high power magnification, 450 X (C) Microphotograph of conidia of *P. palmarum* at low power magnification, 100 X (D) Microphotograph of conidia of *P. palmarum* at high power magnification, 450 X

3.3 Kochs postulate assay

The findings, which are shown in [Fig 3], unmistakably showed that the *Pestalotia* sp. organism was able to begin infect foliage. When infected with *Pestalotia palmarum* (Cooke) Steyaert, the pin pricking approach proved the most successful at causing the GLBD to develop on healthy leaves. Among the three methods, pin prick injury was only effective method for inoculation of the plants. The brushing injury on the rather hard coconut leaves was insufficient to allow *P. palmarum* to successfully enter and colonise, and as a result, no infection or symptom development occurred. The same fungus that was initially isolated, *Pestalotia palmarum* (Cooke) Steyaert, was re-isolated from the tissues of infected coconut leaves, confirming that the disease's symptoms were brought on by that organism. The infectiousness of *Pestalotia* spp., which exhibits the symptoms of GLB on various crops using diverse techniques, has also been demonstrated by numerous researchers. Vyas and Panwar (1974) [14] proved the pathogenicity of *Pestalotia versicolor* (Speg.) Steyaert causing fruit rot of *Zizyphus jujuba* Lamk. by inoculating the fruits by spraying the conidial suspension of the organism over the injured and uninjured fruits and found that only injured fruits developed typical symptoms. Rai *et al*. (1982) [11] proved the pathogenicity of *Pestalotiopsis palmarum* (Cooke) Steyaert causing rot of ber (*Zizyphus mauritiana* Lamk.) by spraying spore suspension on healthy and ripe fruits of ber. Joshi and Raut (1992) [4] proved the pathogenicity of *Pestalotia versicolor* (Speg.) Steyaert causing grey leaf blight disease of clove by inoculating the healthy leaves of the seedlings with spore suspension, after making surface injury so as to facilitate the penetration of fungus. By employing the seedling inoculation approach, Pandey *et al*. (2006) [9] demonstrated the pathogenicity of *Pestalotiopsis mangiferae* (Henn.) Steyaert, which causes stem canker in *Jatropha sativa* L. They also established that in the lack of bark injury, the pathogen unable to enter the plant system and induce disease. These findings are consistent with the results at hand.

3.3.1 Grey leaf blight symptoms produced under inoculated condition by *P. palmarum*

After 15 to 20 days, the surface of the inoculated leaves began to show the typical signs. Initially small, chlorotic spots were observed which later developed into circular brown coloured spots on the inoculated leaves which later increased in size with ashy white center surrounded by dark brown ring and yellow halo [Fig 3b]. Das and Mahanta (1985) [2] studied that *Pestalotia palmarum* (Cooke) Steyaert on coconut caused irregular greyish lesions on the leaflets and in severe cases, several lesions coalesced together to form irregular necrotic patches. Joshi and Raut (1992) [4] studied *Pestalotia versicolor* (Speg.) Steyaert causing grey leaf blight of clove. They observed that initially, brown pin-head like spots appeared on the upper surface of leaves. These brown spots remain always surrounded with yellow halo. Later on, these spots turned white-grey and enlarged in size, coalesced and resulted in blightening of the leaves.

Table 1: Pathogenicity of *P. palmarum* by different inoculation method on leaves of coconut.

Sr. No	Method of inoculation	No. of leaves		Per cent infection
		Inoculated	Infected	
1.	Injury by pin pricking (PP)	4	4	100
2.	Injury by tooth brush (TB)	4	0	-
3.	Spraying of spore suspension	4	0	-
4.	Control	4	0	00

Mango grey leaf spot is consistently caused by *Pestalotiopsis mangiferae* (Henn.) Steyaert, according to Ko *et al*. (2007) [8]. They noticed that the symptoms at first appeared as tiny, brown to yellow dots on leaves. Later, the amorphous dots changed from white to grey and merged to form larger grey areas. Dark borders on lesions were somewhat elevated. On the grey necrotic portions of adult lesions, many black

acervuli measuring 290-328 m in diameter formed. These symptoms were more or less comparable to those reported by Joshi and Raut (1992) [4], Das and Mahanta (1985) [2] and Ko *et al*. (2007) [8],

3.4 Host range assay

Many plant pathogens are highly host specific while others may have wide host range. The pathogens having wider host range, other host may act as alternate host playing an important role in carry over of the pathogen and out-break of the disease. Hence, studies on host range are very important. The data is presented in Table 4.6 and out of four plant species inoculated with *P. palmarum*, typical grey blight symptoms were produced in mango and guava after 10 days of inoculation. In case of mango, initially minute spots were developed on leaves and later these irregular spots turned white to grey in colour (Fig 3c). Symptoms on guava leaves initially appeared as small dark brown spots later turning grey and surrounded by dark brown borders and on guava fruits, tiny water-soaked spots appeared which later became dark in colour and necrotic (Fig 3d.).

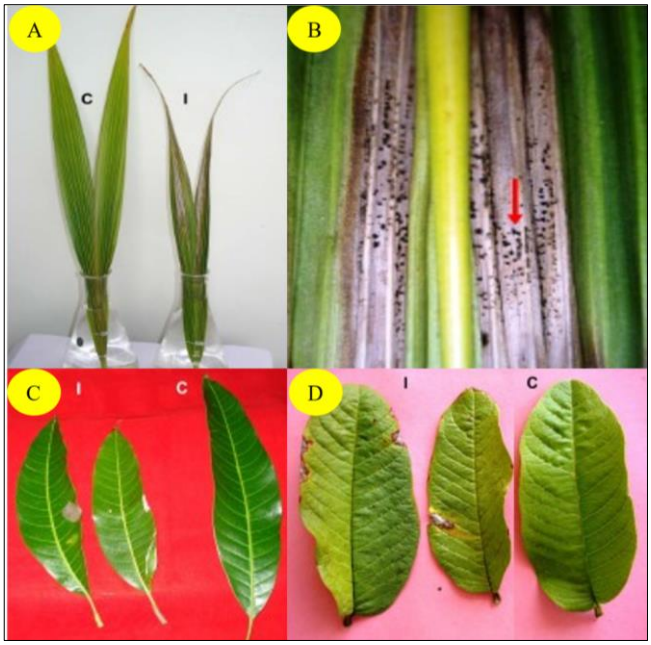

Fig 3: Host range assay (A) Pathogenicity test for coconut GLB caused by *P. palmarum* using pin prick method (B) Close up view of ashy grey blighted area on inoculated PP coconut leaves with development of black acervuli on necrotic area, caused by *P. palmarum* during pathogenicity test (C) Reaction of different test plant against *P. palmarum* on detached leaves/fruit by pin pricking method on mango (I) inoculated leaves (C) Healthy leaf (D) palmarum on detached leaves/fruit by pin pricking method on (I) guava inoculated leaves (C) Healthy leaf

4. Conclusion

The present study proved that the *Pestalotia palmarum* is the main cause of the grey blight of the coconut by the koch's postulate. The authors also concluded that cross inoculation of *P. palmarum* with mango, ber, guava by different methods produced the typical symptoms as produced on the coconut leaves.

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6. Authors Contribution

The research was planned, designed, and carried out by Rokade R. A. The data were evaluated and looked into by Joshi D. M. Someshree Mane provided language improvement advice and edited the manuscript (original draught paper), which was written and reviewed by Khaire P. B. The final version of the paper was authorised by all authors after they had reviewed and amended it.

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