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Discovery of *Curvularia sp.*, *Pantoea sp.*, and *Pseudomonas oryzihabitans*, Associated with Symptoms of Dirty Discolored Grains of Paddy and Panicle Blight in Guyana.

Rajendra Persaud[1]*, Jomeala Gilkes[1], Akeim Casey[1], Darshanie Angela Persaud[2], and Elroy Charles[3]

[1]Guyana Rice Development Board (GRDB), Rice Research Station (RRS), Burma, Mahaicony, East Coast Demerara, Guyana.
[2]The Faculty of Earth and Environmental Sciences, Department of Environmental Studies, University of Guyana, Turkeyen Campus, Greater Georgetown, Guyana.
[3]Faculty of Agriculture and Forestry, University of Guyana, Turkeyen Campus, Greater Georgetown, Guyana.

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***Corresponding Author**
Rajendra Persaud
E-mail: *rajpersaud25@yahoo.com, rpersaud@grdb.gy*

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ABSTRACT

Rice (*Oryza sativa* L.) dirty grains and blighted panicles symptoms were observed on about 1-5% of plants within farmers' fields in Essequibo (region#2,3), Demerara (reg.#4,5) and Berbice (reg.#6) in Guyana. Four samples at maturity stage with Dirty Discolored Grains of Paddy and Panicle Blight were harvested, labeled and place in paper bags in March 2022 from regions#2, 3, 4 and 5 for diagnosis and molecular confirmation. Initial identification to genus-level was done using morphological features and MALDI-ToF. Results of the morphological and molecular sequencing identifies fungal isolates *Curvularia sp.,* with top matches of >99% for *C. lunata, C. dactyloctenicola,* and >98% for *C. inaequalis*. Similarly, sequencing analysis identified *Pantoea* sp. with matches of >99% to several closely related species [including *P. stewartia P. allii, P. ananatis, P. agglomerans* and *P. pleuroti*] and *Pseudomonas oryzihabitans* with top matches of >99% (European Molecular Biology Laboratory (EMBL) database *via* the European Bioinformatics Institute (EBI), (CABI, 2023). Pathogenicity tests were carried out following Koch's postulate and confirmed that *Curvularia sp.*, *Pantoea sp.*, and *Pseudomonas oryzihabitans* were the pathogens responsible for dirty discolored grains of paddy and panicle blight symptoms observed, while the control plants remained healthy and symptomless. To our knowledge, this is the first report that identifies and confirm these 3 pathogens working together and causing the dirty discolored grains of paddy and blighted panicle symptoms in Guyana.

The rice (*Oryza sativa* L.) industry in Guyana is currently the largest agricultural industry in the country. It is the main contributor to export earnings in the agriculture sector and accounted for 18% of Agricultural GDP in 2022. Rice is cultivated primarily along the coastal belt in Essequibo, Demerara and Berbice. During 2022 and 2023 symptoms of dirty grains and blighted panicles symptoms were observed in isolated cases, on about 1-5% of plants within farmers' fields in Essequibo (region# 2 and 3), Demerara (reg.#4 and 5) and Berbice (reg.# 6), in isolated patches in the rice fields across the rice industry in Guyana. Four samples at maturity stage with elongated, light brown lesions on the leaf margins and paddy grains with straw and brownish color, discolored grains with brown spots, distorted, unfilled, partially filled which also appears to be rotted were harvested, labeled and place in paper bags in March 2022 from regions# 2 ,3 ,4 and 5 for diagnosis and molecular confirmation at GRDB, Plant Protection Department (PPD)- Plant Pathology arm at the Rice Research Station (RRS), Burma, Mahaicony, East Coast Demerara for detailed analysis. Likewise, a replicate of the said four samples collected were packaged and sent to the Center for Agriculture and Bioscience International (CABI or CAB International), United Kingdom (UK), Diagnostic and Advisory Service department for assistance with the further identification and molecular confirmation of the complex group of microorganisms observed. Initial identification to genus-level was done using morphological features and MALDI-ToF. The isolation process begins with the discolored grains being removed from the panicles, surface was sterilized for 1.5-3 minutes in sodium hypochlorite (NaOCl) prior to plating on to Tap Water Agar (TWA) plates; Placed into a damp chamber to induce sporulation; Subcultures of the resultant fungal growth were then plated onto Plate Count Agar (PCA) and were identified to genus-level using morphological features, and two dominant isolates were selected and submitted for the molecular identification. Similarly, discolor grains were surface cleaned with ethanol, then crushed in sterile distilled water and streaked onto Trypticase Soy Agar (TSA) plates and placed into a damp chamber; Subcultures of bacteria were plated onto Nutrient Agar (NA) and identified initially using Matrix-Assisted Laser Desorption Ionization, Time-of Flight (MALDI-ToF) mass spectrometry. Likewise, three dominant bacterial isolates were selected and submitted for molecular identification/ sequencing analysis.

Results of each morphological identification found *Curvularia sp.*, *Pantoea sp.*, and *Pseudomonas sp.,* to be the most predominant microorganisms present of the discolored paddy grain samples analyzed. The molecular sequencing for the two fungal isolates identified both as being *Curvularia sp.,* with top matches of >99% were made to several species including *C. lunata, C. dactyloctenicola,* and >98% for *C. inaequalis* for the sequencing results. *Curvularia* species have a worldwide distribution, occurring as pathogens or saprobes of a wide range of plants. *Curvularia. lunata* is well documented in literature for its involvement in causing dirty panicles of rice (Persaud et al., 2019 and 2022) and works with other microorganism species from different genera to cause the symptoms described (Seephueak et al., 2019).

Similarly, sequencing analysis of the three bacterial isolates identified *Pantoea stewartia*, *Pantoea* sp. with matches of >99% to several closely related species [including *P. allii, P. ananatis, P. agglomerans* and *P. pleuroti*] and *Pseudomonas oryzihabitans* with top matches of >99% by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database *via* the European Bioinformatics Institute (EBI). *Pantoea* isolates have been known to be isolated from habitats including plants, humans and environmental sources. Many different *Pantoea* species have been isolated from a variety of plants including maize, grasses and rice and there are mixed reports of both pathogenicity and beneficial properties (Doni et al., 2021). Both subspecies of *P. stewartii* (*indolgenes* and *stewartii*) are phytopathogenic and responsible for wilts, blight and leafspots. The *Pantoea* genus has also been described as the causative agent of grain discoloration since 1983, when it was first reported in Japan (Azegami 1983), China (Xie 2001; Hong et al. 2002; Yan et al. 2010), West Africa (Kini et al., 2020), India (Logeshwari et al., 2023) and Brazil (Horn et al., 2023). The disease contributed to severe losses in rice production, as much as 75%, due to grain weight reduction, floret sterility, strands reduction, inhibition of seed germination and year-to-year transmission on account of the seed borne pathogen (Trung et al. 1993). Symptoms initially include light, rusty, water-soaked lesions on the lemma or palea, which later turn brown, causing grain discoloration and abortion (Yan et al. 2010). Likewise, *Pseudomonas oryzihabitans* species has been recently reported as pathogen of rice, causing a panicle blight and grain discoloration with different symptoms to *B. glumae* (Hou et al., 2020).

The different bacterial and fungal isolates isolated from the discolored portions of the rice grains found fungi, *Curvuularia* sp. (*C. lunata*) and bacteria species *Pantoea* sp. (especially *Pantoea ananatis)* and *Pseudomonas oryzihabitans* working together and responsible for the discoloration of grains, as these pathogens are known to be associated with '*discolored rice panicles and leaves*' and are known to work in conjunction with other fungal and bacterial species to cause grain discoloration and panicle blight symptoms (Azizi et al., 2020).

Pathogenicity testing was carried out following Koch's pustulates utilizing the protocol as described by (Yan et al., 2010 and Hou et al., 2020) to determine if any of the above pathogens were responsible for the symptoms observed. For pathogenicity test, eighteen pots (30x30x27cm) filled with soils, planted with Rustic

cv. Grow outdoor between 30 to 37^{o}C at >75% RH. At booting stage nine pots were inoculated by injecting the panicle buds with spore of *Curvularia* sp. (10^{6}/ml) and bacteria suspension of *Pantoea* sp. and *Pseudomonas oryzihabitans* (10^{8} CFU/ml, each). Water was used as control. At 5-7 days after inoculation (DAI), water-soaked, light brown lesions start appearing on leaves, and the heading rice showing light-brownish color on grains 7-10 DAI. Later at 14-21 DAI, the entire grains become discolored with brown spots, distorted, unfilled, partially filled like the symptoms observed in farmers' fields in region #2, 3, 4 and 5. In contrast, the control plants remain healthy and symptomless. Hou et al., (2020) Kini et al., (2020), and Persaud et al (2019) reported similar findings. Re-isolation yielded *Curvularia sp.*, *Pantoea sp.*, and *Pseudomonas oryzihabitans* from the inoculated plants. To our knowledge, this is the first report that identifies and confirm these 3 pathogens causing the dirty discolored grains of paddy and blighted panicle in Guyana.

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DECLARATIONS STATEMENTS

Competing interest and Fundings: The authors have no relevant financial or non-financial interests to disclose.

Data availability*:* The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions: RP: designed and executed the study, analyzed the data, and drafted the paper. All other authors provided editing support, technical advice, read, and agreed with the content of the paper.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare that there is no potential conflict of interest to report.

Research involving Human Participants and/or Animals: The authors declare that the current research did not involve human participants or animals as test materials.

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SUPPLEMENTARY INFORMATION

Molecular Methods and Basis for ID (CABI or CAB International, 2023):

Methods utilized for Molecular Identification of Bacteria, Fungi and Yeasts:

All original samples are subjected to a purity check.

Molecular assays are carried out on each sample using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] is subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA).

Following DNA extraction, Polymerase Chain Reaction (PCR) is employed to amplify copies of the rDNA in vitro.

The quality of the PCR product is assessed by undertaking gel electrophoresis.

PCR purification step is carried out to remove unutilised dNTPs, primers, polymerase and other PCR mixture compounds and obtain a highly purified DNA template for sequencing. This procedure also allows concentration of low yield amplicons.

Sequencing reactions are undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing.

Removal of excess unincorporated dye terminators is carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK).

Modules containing prehydrated gel-filtration resin are optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal is followed by suspension of the purified products in highly deionised formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation.

Samples are loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide.

Following sequencing, identifications are undertaken by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI).

Molecular Identifications:

Basis for level of identification

All identifications are based, unless otherwise stated, on matches to sequences published in peer-reviewed literature or matches to a validated type strain.

Species level identification

Identification is provided to species level, or where appropriate to species group for bacteria, where matches of 99-100% identity are obtained and for fungi, where matches of 97-100% identity are obtained, provided that matches include a sequence derived from type or other validated culture and when there is a clear sequence distinction between taxa.

Clade level identification

Identification is given to clade level for bacteria and fungi where matches of 97-100% identity are obtained to more than one species within a genus belonging to a single published clade and there is no clear sequence distinction between taxa.

Genus level identification

Identification is given to genus level for bacteria where matches are lower than 99-100% (i.e. 97-98% identity) to a single taxon and for fungi where matches are lower than 97-100% (i.e. 95-96% identity) to a single taxon.

Also, for both bacteria and fungi, identification is given to genus level where matches of 97-100% identity are obtained to more than one species within a genus and there is no clear sequence distinction between taxa.

Family level identification

Identification is provided to family level for bacteria and fungi where matches of 95% identity or above are obtained to more than one genus, but all belong to one family.

Order level identification

Identification is provided to order level for bacteria and fungi where matches of 95% identity or above are obtained to genera of more than one family,

Non identified samples

Where no identification can be provided, the sample report states why this is the case.

Methods outside the scope of accreditation

Morphological Identification

Identification of fungi by morphological analysis involves preparation of subcultures on diagnostic media. Colony morphology of the original sample and subcultures is examined. Slides are prepared and microscopic analysis of sporulating structures is undertaken at x400 magnification. Identification is based on comparison with published taxonomic descriptions.

Additional information for E1006

E1006001: *Pantoea sp.*

Top matches of >99% were made to members of this genus and included several different species. The validated type strain sequence of P. allii [AY530795] gave a match of 100%, and P. pleuroti [KJ654341] gave a match of 99.7%. Sequences derived from the validated type strain of P. ananatis ATCC 33244 [U80196] gave a match of 100%, and P. agglomerans [DSM 3493] also gave a match of 99.7%.

E1006002: *Pseudomonas oryzihabitans*

Top matches of >99% were made to sequences derived from the validated type strain of this species [NBRC 102199:], and [LMG 7040:] giving matches of 99.5% and 99.6% respectively.

E1006003: *Pantoea stewartii*

Top matches of >99% were made to sequences ascribed to Pantoea stewartii and included the validated type strain sequence of Pantoea stewartii subsp. indologenes LMG 2632 [Y13251] with a match of 99.1%, and a sequence derived from a validated type strain of Pantoea stewartii subsp. stewartii LMG 2715 [Z96080] gave a match of 98.9%.

E1006004: *Curvularia sp.*

The ITS sequence obtained from this sample showed top matches at >99% identity to members of the genus Curvularia. The best matches included sequences from type strains published in peer-reviewed literature, e.g. 100% identity to sequence MF490815 from the type strain of Curvularia dactyloctenicola (CPC 28810) and 99.8% identity to sequence MF490814 from the type strain of C. chiangmaiensis, both of which have been published in published in Tan Y.P. et al. (2018)

E1006005: *Curvularia sp.*

Using FASTA with the EBI database, top matches to fully named and published sequences were to members of the genus Curvularia with matches at >98% identity. These included sequences published in peer-reviewed literature, e.g. 98.5% identity to Curvularia inaequalis sequence MH856096 from reference culture collection strain CBS 102.42 and 98.5% identity to Curvularia protuberata sequence MH864531 from reference culture collection strain CBS 127342, both of which have been published in Vu, D. et al. (2018).

Sequence data for the five selected microorganism for molecular characterization and identification

Sequence data: E0001006
E0001006001 partial 16S sequence:
>E1006001BR_003_2022-05-12_G01.ab1
CTGCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGC
TTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGC
TGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGGGCCGTTACCCCGCCTACTAGCTAATCCCATCT
GGGTTCATCCGATAGTGAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCCACCGT
TTCCAGTGGTTATCCCCCTCTATCGGGCAGATCCCCAGACATTACTCACCCGTCCGCCACTCGTCACCCGA

E0001006002 partial 16S sequence:
>E1006002BR_004_2022-05-12_H01.ab1
GGTATTCGCTATGAGCCCTTCCTCCCAACTTAAAGTGTTTTACGACCCGAAGGCCTTCTTCACACACGCGGCAT
GGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCA
GTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCTACCAACTA
GCTAATCCGACCTAGGCTCATCTAATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGG
TATTAGCGTTCCTTTCGAAACGTTGTCCCCCACTACTAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTG
AATCGAGGAGCAAGCyCCTCTCATCCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCA

E0001006003 partial 16S sequence:
>E1006003BR_001_2022-05-12_A02.ab1
GCTGAGGTTATTAACCTCAGCACCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGC
GGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGT
GTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGGGCCGTTACCCCG
CCTACAAGCTAATCCCATCTGGGCACATCCGATGGTGTGAGGCCCGAAGGTCCCCCACTTTGGTCTTGCGACGT
TATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCGGGCAGTTTCCCAGACATTACTCACCCGTCC
GCCACTCGTCACCCGAGGAGCAAGCTCCTCTGTGCTACCGTCCGACTTGCATGTGTTAGGCCTGCCGCCAGCG
TTCAATCTGA

E00010006004 partial ITS sequence:
>E1006004FR_001_2022-05-12_E01.ab1
AGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCA
CCAAATAAACCTTTTTTATGCAGTTGCAATCAGCGTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCC
TCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTCGCCCAAAGACTCGCCTTAAAGTGATTGGCAGCCGG
CCTTTCTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCCATCAGCAAAACGGCAATCCATCAAGCCTCCTTCTC
ACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC

E0001006005 partial ITS sequence:
>E1006005FR_002_2022-05-12_F01.ab1
GCCCGCGGCTGGTGTTTCCCCTTCTCGGGAGGCGCCAGTTGGCGGACGCTGGACTATTTTATTACCCTTGTCTT
TTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCACCAGGACCACACCATAAACCTTTTGTATGCAGTTGC
AATCAGCGTCAGTACAACAAATGTAAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
GAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGT
TTTTTTTTTT

Images of the discolored grains observed under field conditions in Essequibo and Demerara (Region # 2, 3, and 4, Guyana) *(Photo: GRDB, 2023).*

Images of the discolored grains at harvest in Essequibo and Demerara (Region # 2, 3, and 4, Guyana) *(Photo: CABI, 2023)*

Reference:

Center for Agriculture and Bioscience International (CABI or CAB International). 2023. Final report of analysis. Diagnostic and Advisory Service. United Kingdom (UK).

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