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Pantoea dispersa: A Novel Threat to Rice Cultivation in India

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ABSTRACT: Approximately more than 50% of India's population relies on rice (*Oryza sativa*) for their daily nutrition, making it the country's most important crop. The rice plant is susceptible to a number of important diseases, including blast, brown spot, sheath blight, false smut, sheath rot, stem rot, bacterial leaf blight, leaf streak, bakane disease, and khaira disease. A newly emerging disease of rice that exhibits symptoms similar to BLB has only lately been identified in major parts of Asia, such as China, Malaysia, Korea, and Thailand, as well as in other nations, such as Germany, Turkey, Togo, Brazil, and Venezuela. In the past, *P. ananatis* was discovered to be the pathogen that was causing rice leaf blight in northern India. In this study, a new species of *Pantoea* was isolated from diseased seeds and plant parts causing leaf and grain blight of rice from Odisha, India. To identify the bacterial pathogen, typical symptoms were described, and morphological, biochemical, and molecular characterization using the 16s r RNA region were performed to confirm the bacteria as *Pantoea dispersa*.

Keywords: *Pantoea dispersa*, emerging disease, grain blight, 16s rRNA, India.

INTRODUCTION

Rice (*O. sativa*) is India's most significant crop, covering 25% of the farmed land and feeding half of the population. India produces 125.0 Mt of rice on 45.50 Mha at 4.1 T/ha. Bacteria, fungus, and nematodes generate considerable economic losses to the rice production in India. Blast, Brown Spot, Sheath Blight, False Smut, Sheath Rot, Stem Rot, Bacterial Leaf Blight, Leaf Streak, Bakane and Khaira Disease are major rice diseases in India. Bacterial leaf blight is one of the most damaging rice diseases in tropical and temperate regions, particularly in Asian countries (Naqvi, 2019). The worst and longest-lasting rice disease in Asia is known to be bacterial leaf blight, which is brought on by the bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo) (Naqvi, 2019) and it is one of the most destructive bacterial diseases affecting the major rice growing regions of the world (Yuan *et al.,* 2010). In another investigation, another bacterial pathogen has been reported, namely *B. glumae,* which caused spikelet sterility, browning of grains, grain weight loss, floret sterility, and seed germination inhibition (Ham *et al.,* 2011; Pedraza *et al.,* 2018).

New and re-emerging plant diseases limit global agricultural output. A new emerging rice disease with BLB-like symptoms has recently been reported in major parts of Asia like China, Malaysia, Korea, Thailand and other countries like Germany, Turkey, Togo, Brazil, Venezuela (Lee *at al.,* 2010; González *at al.,* 2015; Toh *et al.,* 2019). *P. ananatis* is the main pathogenic species for this disease, however *P. stewartii, P. agglomerans, P. dispersa*, and other species can also cause it. Eventually, this *Pantoea* genus will be considered a global threat to rice production, resulting in significant yield and quality losses (Azizi *et al*., 2020). In some countries like Germany, Togo, Malaysia, and Thailand, two species complexes caused the illness, illustrating its complexity. Previously In September to October 2021 blight disease on the rice leaves and blighted panicles were observed in the paddy cultivar Swarna Sub-1, in the paddy fields of Ambabhona Village (21°34'34.3"N 83°28'08.2"E) of Bargarh district, Odisha state, India. Lesions appeared 1stas water-soaked spots with yellowish or brownish in colour, eventually become necrotic causing leaf blight of rice. Grains are also affected in which rusty, brownish water-soaked lesions are appeared on the lemma or pale a of the grains and gradually becomes blighted and no grain filling occurred, leads to chaffy seed formation. In this study, the occurrence of *Pantoea dispersa* causing leaf and grain blight of rice plants in India described in details for the first time. There have been no previous reports of *Pantoea dispersa* causing rice disease in India. To identify the bacterial pathogen, typical symptoms are described and morphological, biochemical and

molecular characterization using the 16s r RNA region were performed on the pathogen.

MATERIALS AND METHODS

Collection and isolation of pathogen. The infected plants and their parts showing symptoms of disease at the field were critically observed for symptoms of the disease. The surface of the tissues were made infection free with 70% ethanol for a short period of time, followed by a 1-minute soak in 1% sodium hypochlorite solution, and then washed in sterile distilled water and the resulting sterilized samples were placed on to King's Bagarmedium and further incubated in the growth chamber rat 28 ± 1ºC. The Petri plates were examined after 48 h for the recovery of the bacterium. Bacteria form colonies in 2 days. Single colonies were usually multiplied on King's B agar medium. The culture was preserved by transferring it to King's B agar medium in culture tubes, and it was then refrigerated for additional investigation. A semi-selective medium originally created by Kini *et al.* (2019) was used to isolate and distinguish the *Pantoea* from other bacteria. It constituted 1 liter of sterile distilled water (pH around 7.1), 10 g peptone, 8.5 g of sodium thiosulfate, 13.5 g of agar, 65 g of NaCl, 0.001 g of crystal violet and 10 g of sucrose. For scanning electron microscopy bacterial cells were harvested by centrifugation (2500 rpm for 10mins at 4°C) and washed the cell pellets twice using IXPBS. Cells were diluted up to 10^7 CFU/ml using IXPBS. 200µl of diluted bacterial suspension was spread over a sterile aluminum foil (5cm × 5cm). The foil was kept inside the laminar hood for ~1hr for complete drying. Dried foil was kept in 2.5% glutaraldehyde for o/n at 4°C for fixing of cells. Cells were dehydrated with a series of ethanol treatment (30%, 60%, 70%, 80%, and 90%) for 10mins each. Finally, cells were treated with 100% ethanol for 30 min. Kept inside the laminar hood for ~30-45mins for complete drying. Then gold-palladium particles were sputtered over the cells by an automated sputtering machine and visualized via SEM.

Biochemical Identification. The Gram reaction was seen using the technique outlined in Bergey's Manual of Determinative Bacteriology (Buchanan and Gribbons 1974). On a clear, dry glass slide, a thin smear was prepared and allowed to air dry before, being fixed with mild heat. The smear was soaked with Gram's Crystal Violet for 1 minute before draining the stain with Gram's Iodine. Gram's Decolorizer was applied after 1 minute, until the blue dye no longer flowed from the smear. Following washing with tap water, 0.5% w/v Safranin was added and allowed to sit for 1 minute. The slide was air dried after final rinsing and viewed using an oil immersion objective microscope.

The VITEK2 automated microbiology system was used for biochemical analysis, and the gram-negative reagent cards with 64 wells that each containing an independent test substrate was used. Inserting the transfer tube into the appropriate suspension tube, a test tube containing the microorganism suspension is put on a specific rack (cassette), and the identification card is inserted into an adjacent slot. The filled cassette is carefully placed inside the vacuum chamber station. The organism suspension is pushed into micro-channels that fill all of the test wells through the transfer tube after applying vacuum and re filling the station with air. The GNcards designed to measure carbon source utilisation, enzyme activity, and resistance (Pincus, 2006). The whole results of the identification process may be obtained in within 10 hours and the organism was identified from the VITEK-2 database.

Molecular Characterization. DNA was extracted utilizing Zymo Research Quick-DNATM Bacterial Miniprep Kit, Catalog No. D6005. In order to separate the amplified DNA, it was electrophoresed in a 0.8% agarose gel using 1 TAE buffer at 50V for 30 to 45 minutes, or until the DNA fragments moved smoothly. The 16s rRNA analysis was carried out by using following sets of primer Forward 27F(5'AGAGTTTGA TCCTGGCTCAG3') and reverse 1492R (5'TACGGTT ACCTTGTTACGACTT3'), The template was warmed at 95 °C for 5 minutes to denature it. 39 cycles of denaturation (for example, 30 seconds at 95 degrees, 45 seconds of annealing, and 1 minute of elongation at 72 degrees Celsius) were then conducted, with the ultimate extension reaching 7 minutes at 72 degrees Celsius. Using 0.5x tris-acetate-EDTA (TAE) buffer, the amplicons have been analyzed on 1.5% agarose gel. Sequencing was carried out and the 16S rRNA gene consensus sequence was generated from forward and reverse sequencing data using aligner software. The NCBI GenBank 'nr' database was searched using the 16S rRNA gene sequence. Based on their greatest identity score, sequences were selected and aligned using the multiple alignment program Clustal W. The distance matrix was generated from the RDP database, and MEGA 11 was used to create the phylogenetic tree.

Pathogene city Test. Pathogenicity test was carried out using the method described by Kini *et al.* (2020). Among different rice cultivars on the basis of symptoms development and disease scoring from varietal screening, Swarnasub 1 was selected for the pathogenicity test. Seeds of Swarnasub b 1 variety were collected from certified sources in order to ensure they were free from seed borne pathogens. Seeds pre-soaked overnight were sown in nursery beds and moisture was maintained for their proper germination. Thirty days old rice seedlings were transplanted in 20 cm diameter pots filled with potting compost (121.6 °C for 20 min) under semi-controlled circumstances in growth chambers with 10 hours of light, 30°C during the day and 22°C at night, and 75% relative humidity. Three rice seedlings were transplanted in each pot. Five such replications were made *i.e.* total of 15 seedlings. For the best plant development, urea was used as per recommended dose. Bacterial strains were spread that were kept in a freezer at -20°C on prepared King's B agar plates and incubated at 28°C for 1–2 days. The cells were

scrapped off the plates and were put in a bowl of sterilized ultrapure water. A spectrophotometer was used to set the concentration of this solution to 10^7cells/ml (OD = 600 nm).Then, after 15 days of transplanting, a needleless syringe was used to pump 1 to 2 ml of the bacterial suspension inoculum into the 3 to 4 fully grown leaves. Leaves were injected about 3 to 5 cm below the leaf tips. Inoculation on the leaves was done by pressing the mouth of the needle against the leaf surface. Plants in the control group were sprayed with sterile distilled water. Leaf surface was gently wiped with a sterilized paper towel to remove the excess liquid from the leaf surface. Symptom development was observed on regular basis.

Assessment of different methods of inoculation. The study compared various methods of inoculation to standardize the best method for artificial disease incidence. Seed inoculation involved subjecting 20 healthy rice seeds to a bacterial solution containing 108 CFU per milliliter of pathogenic bacteria. The seeds were then infected and subjected to a vacuum for 10 minutes. The seeds were then planted in sterilised soil and incubated in a controlled environment. Soil inoculation involved mixing 10g of chopped paddy straw in 1 kg of sterilized soil. Root dip method involved soaking rice roots with a bacterial suspension for 2 hours, then replacing the suspensions with sterile distilled water. Infiltration involved injecting the inoculum into the plant's boot leaf, infiltrating slowly and incubating in warm and humid conditions. Spray inoculation involved spraying a bacterial suspension on healthy paddy plants, maintaining turgidity of leaves with a humidifier.

Longevity of bacterium in naturally infected seed at *in vitro.* Seeds were stored at 14-30ºC and examined monthly for bacterial recovery. Bacteria from contaminated seeds were observed on sterilised culture plates. The seeds were then planted in the cropping season under controlled glass conditions to assess the disease progression from the stored seeds. The study aimed to determine the effectiveness of storage conditions.

RESULTS

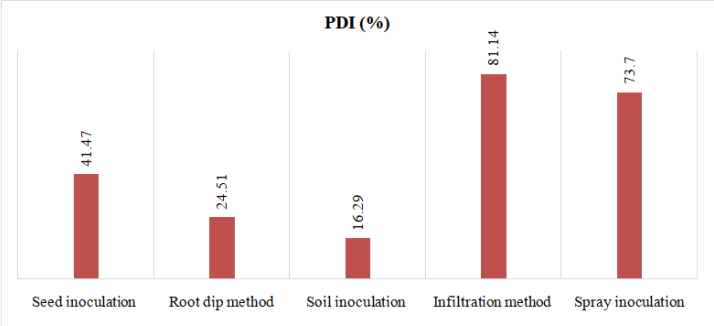
Isolation of pathogen. The causal organism isolated from the diseased leaf tissue and the affected panicles was found to be a bacterium. The colony of the isolate in the King's B agar medium was a regular in shape with a smooth surface, margin entire, raised elevation and slightly yellowish in color. The isolate was named as BA 1 and was found to be Gram –ve, aerobic and rod shaped under the light microscope. In *Pantoea* specific agar after 72hrs the bacterium colonies were greyish to pale yellowish in color, coalesced and highly viscous in nature.

Biochemical Characterization. The VITEK-2 analysis confirmed that the bacteria as *Pantoea dispersa* at 97% probability which is gram -ve in nature and showed positive results for D cellobiose, β Galactosidase, D Glucose, D Mannitol, D Mannose, Sucrose, D Trehalose, L Lactate, Succinate, Phosphatase, L-Pyrrolydonyl-arylamidase, beta-n-acetyl-glucosaminidase, gamma-glutamyl-transferase, D-maltose, L-prolinearylamidase, Tyrosinearylamidase, citrate (sodium), 5-keto-D-gluconate, succinatealkalinization, courmate and negative results for H_2S production, β Glucosidase, D Maltose, Lipase, Urease, D Sorbitol, Mannitol, L Lactase, adonitol, L-arabitol, Glutamyl Arylamidasep NA, beta-xylosidase, 27palatinose, malonate, alpha-glucosidase, alpha-galactosidase, ornithinedecarboxylase, glycinearylamidase, lysinedecarboxylase, decarboxylasebase, L-histidineassimilation, and Glu-Gly-Arg-arylamidase.

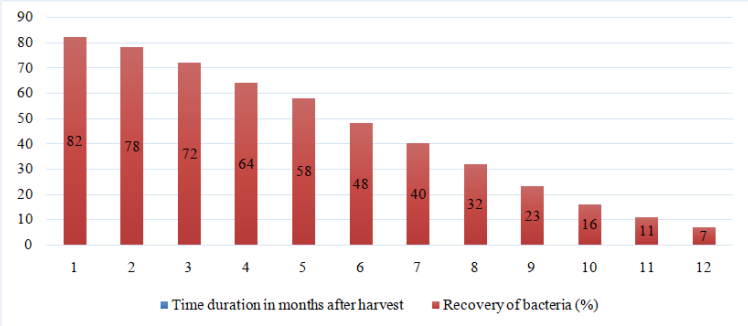
Molecular Characterization. The 16s rRNA analysis using primer 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'TACGGTTACCTTGTTACGACTT3'), showed similarity of the isolate with *Pantoea dispersa* and it was submitted to Genbank with accession no OP753655.1. The sequence showed 100% nucleotide identity with *Pantoea dispersa* strain MT646430.1.

Pathogenicity test. In susceptible reaction water soaking spots appeared 4-5 days after inoculation, mainly at the infiltrated spots of the leaves and later it turned in to yellowish or brownish in colour and eventually became necrotic after 15-20 days after inoculation, and in the course of time, the aforementioned symptoms appeared on the grains also. The bacterium was re-isolated from the diseased samples and was confirmed to be *Pantoea dispersa*after biochemical and 16s rRNA analysis. After panicle emergence, individual grains werealso affected showing rusty, brownish to greyish water-soaked lesions on the lemma orpalea andgradually became blighted and no grain filling occurred, leading to chaffy grain formation.

Assessment of different methods of inoculation. Under net house conditions, plants exhibited symptoms after 8 days when subjected to various techniques of artificial inoculation with the *Pantoea dispersa* isolate BP1. The following methods were documented: (i) seed inoculation, (ii) soil inoculation, (iii) root dip method, (iv) infiltration method, and (v) spray method. The infiltration approach and spray inoculation method resulted in the highest per centage of disease incidence and equivalent susceptibility in terms of panicle infection (81.14), followed by the spray inoculation method (73.70). The results of the study indicate that the soil inoculation method (16.29) exhibited the lowest efficacy in initiating disease under controlled artificial inoculation settings.



Graph 1. Different methods of inoculation on disease expression.



Graph 2. Longevity of bacterium in naturally infected seed.

Longevity of bacterium in naturally infected seed at *in vitro*. The study examined the recovery of bacterium from infected seeds collected from the field from November 2021 to October 2022. The maximum recovery was observed after harvest, with 82% of seeds showing bacterial recovery. Seedlings from stored seeds showed panicle blight symptoms at panicle emergence stage. After 12 months, bacterium recovery from stored infected grains was 7%.

DISCUSSION

Because of changes in global climate, excessive monoculture, geographic expansion, changing land use, and the use of immunosuppressive medications in agricultural techniques, the scope of new emerging disease threats has grown rapidly over the last two decades (van Rhijn and Bromley 2021). Several researchers found that using *Pantoea* strains boosted rice growth and output owing to their favourable impacts and ability to colonise rice (Dutkiewicz *et al.,* 2016). *Pantoea* may promote rice development primarily by direct nutritional acquisition and phytohormone synthesis, as well as indirect suppression of rice plant diseases, establishing broad-spectrum resistance, or reducing abiotic stress (Ortmann *et al.,* 2006; Sun *et al.,* 2020; Sun *et al.,* 2020; Yang *et al.,* 2020 ; Lu *et al.,* 2021). The maximum recovery was observed after harvest, with 82% of seeds exhibiting bacterial recovery. After 12 months, bacterium recovery was only 7%. According to Shahjahan *et al.* (1998), it was observed that the bacterium's recovery gradually decreases after a period of six months. Consequently, when these seeds are sown, they display indications of panicle blight on the plant. Again, the infiltration and spray inoculation methods reproduced highest disease incidence and panicle emergence susceptibility (81.14), followed by the spray inoculation method (73.70). The soil inoculation method (16.29) had the lowest efficacy in initiating disease under controlled artificial inoculation settings, followed by the root dip approach (24.51). Several studies conducted by Azegami *et al.* (1987); Tsushima *et al.* (1996); Nandakumar *et al.* (2009); Wubneh and Bayu (2016) have provided confirmation of the aforementioned findings. These studies have demonstrated that there exists variability in the development and manifestation of diseases, depending on the methods employed for pathogen inoculation. Again, Multiplex PCR tools—allowed the precise and concurrent identification of the three main plant-pathogenic *Pantoea* spp. (*P. agglomerans, P. ananatis, and P. stewartii*).

However, several studies have shown that the emerging rice disease *Pantoea* has a significant detrimental effect on rice output, leading to significant economic losses (Doni *et al.,* 2019; Azizi *et al.,* 2020). *Pantoea* clearly plays a contentious role in rice health, making it a current study focus. Due to change in global climatic conditions and alternation of genetic diversity among the *Pantoea* isolates the virulent strains become more prominent which affect the rice plants.

Fig. 1. Severely affected panicles.

Fig. 2. Infected seed showing bacterial growth.

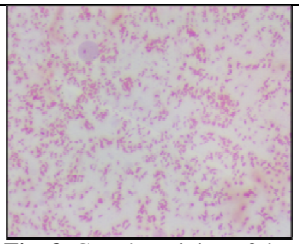


Fig. 3. Gram's staining of the bacteria.

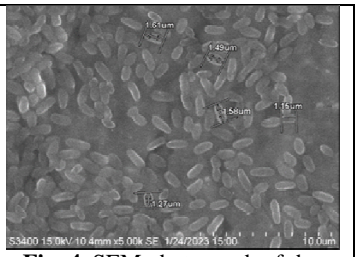


Fig. 4. SEM photograph of the bacteria.

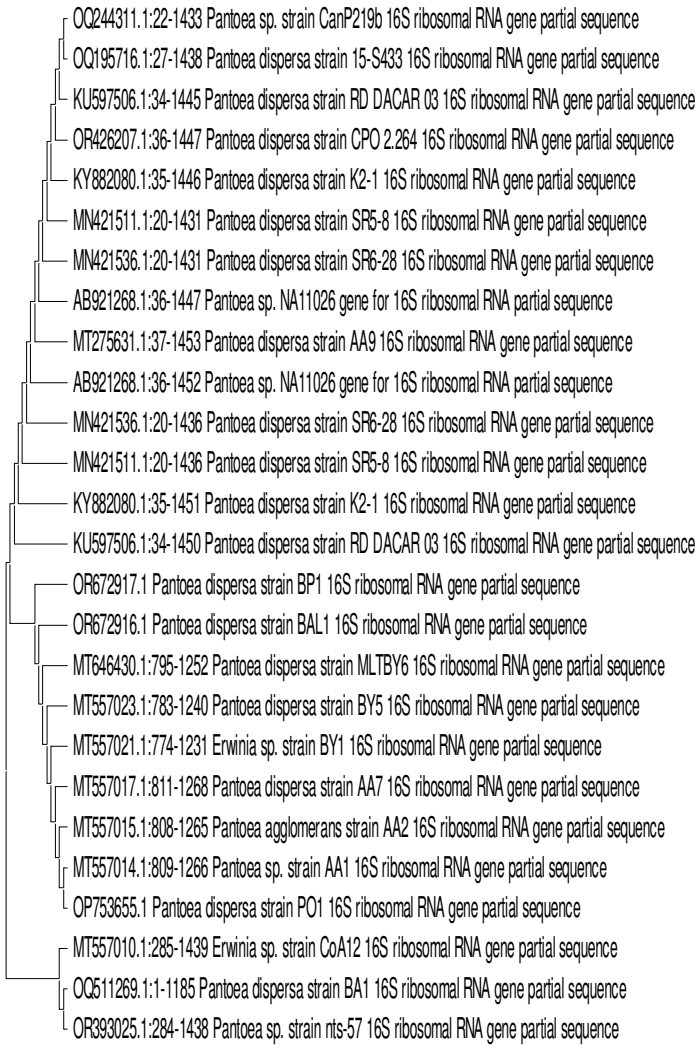


Fig. 5. Phylogenetic tree of the *P. dispersa.*

CONCLUSIONS

In conclusion, this is the detailed report of *Pantoea dispersa*-caused rice leaf and grain blight in India with their biochemical and molecular characteristics. The rising number of reports of disease on novel hosts in various regions of the globe validates the idea that this pathogen is an emerging pathogen. In recent times, there has been a growing focus on comprehending the mechanisms behind the many functions of *Pantoea* in relation to rice plants. Undoubtedly, this study domain merits more investigation.

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Conflict of Interest. None.

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