Biological Forum – An International Journal **15(1): 674-677(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Detection of *Pantoea* spp., an Emerging Pathogen of Rice through Multiplex PCR System

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(Received: 05 December 2022; Revised: 28 December 2022; Accepted: 03 January, 2023; Published: 21 January, 2023)
(Published by Research Trend)

ABSTRACT: The recent fluctuations in climate have introduced a novel challenge to rice cultivation, characterized by the occurrence of panicle blight and incomplete grain filling. A new causative agent was classified under the taxonomic genus *Pantoea* have been confirmed as the causative agents of leaf and grain blight disease in different rice growing regions of Odisha. A multiplex polymerase chain reaction (PCR) assay was developed with the purpose of differentiating various species within the *Pantoea* genus. A set of polymerase chain reaction (PCR) primers was designed to selectively detect the housekeeping genes, namely in fB, gyrB, and rpoB, in species *P. agglomerans, P. ananatis,* and *P. stewartii.* These primers were designed to have varying base pair compositions. A separate set of primers was developed to facilitate the identification of the at pD gene, a gene that is typically found in all *Pantoea* spp. Additionally, a universal set of primers was designed to amplify the 16S rRNA region, a region that is common among all the bacterial species. In the mPCR system, a total of five isolates were taken, namely BA1, BAL1, BP1, which were identified as *Pantoea agglomerans,* NP1, identified as *Pantoea dispersa,* and one *Xoo* bacterium. The polymerase chain reaction (PCR) was conducted, resulting in the formation of discrete bands measuring 1.5 kilobases (kb) for each of the isolates. Distinct bands were observed at a length of 330 base pairs (bp) during the amplification of the at pD gene in the four *Pantoea* isolates, with the exception of *Xoo*. The amplification of the inf B gene was conducted on the isolates of *Pantoea agglomerans*, resulting in the formation of distinct bands at 730 base pairs (bp) for the isolates BA1, BAL1, and BP1. The method employed in this study involved the utilisation of multiplex polymerase chain reaction (mPCR) to simultaneously amplify bacterial DNA using multiple sets of primers. The resulting distinct bands observed in the gel electrophoresis analysis allowed for the differentiation of various isolates, as well as the identification of *Pantoea* bacteria at the species level.

Keywords: *Pantoea agglomerans, Pantoea dispersa,* mPCR, *Xoo (Xanthomonas oryzae* pv. *oryzae.*

INTRODUCTION

The genus Pantoea was initially described in 1989 and has since been taxonomically classified as a member of the Erwiniaceae family (Adeolu *et al.,* 2016; Gavini *et al.,* 1989). The *Pantoea* genus comprises a diverse group of over 25 species, some of which have been identified as pathogens affecting various crop plants, including rice (Kini *et al.,* 2018). This new emerging rice disease with BLB and panicle blight like symptoms has recently been reported in major parts of Asia like China, Malaysia, Korea, India, Thailand, and other countries like Germany, Turkey, Togo, Brazil, and Venezuela (Doni *et al.,* 2019). Three species of *Pantoea,* specifically *Pantoea agglomerans, P. ananatis, P. dispersa* and *P. stewartii,* have been consistently found in symptomatic rice samples worldwide (Toh *et al.,* 2019; Cother *et al.,* 2004; Egorova *et al.,* 2015; Gonzalez *et al.,* 2015; Kini*e t al.,* 2017; Lee *et al.,* 2010; Mondal *et al.,* 2011; Yan *et al.,* 2010). The recent fluctuations in climate have introduced a novel challenge to rice cultivation, characterised by the occurrence of panicle blight and incomplete grain filling. The disease exhibits sporadic incidence within select rice-growing districts of Odisha, leading to significant economic losses for the affected farmers. A set of PCR primers that specifically detect the housekeeping genes such as inf B, gyr B, and rpo B of *species P. agglomerans, P. ananatis,* and *P. stewartii* with different base pairs was designed (Kini *et al.,* 2021). The introduction of this novel molecular diagnostic tool is anticipated to facilitate precise identification of prominent plant-pathogenic species belonging to the genus Pantoea. Multiplex PCR tools—allowed the precise and concurrent identification of the three main plant-pathogenic *Pantoea* spp. *(P. agglomerans, P. ananatis, and P. stewartii) (*Bangratz *et al.,* 2020). This novel diagnostic method will be valuable for phytosanitary services in regular *Pantoea* spp. diagnoses

in any kind of sample (for example, leaves, seed, soil, or water and other planting materials).

MATERIALS AND METHODS

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 tissue was 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 onto King's B agar medium and further incubated in the growth chamber at 28±1ºC. The Petri plates were examined after 48 h for the recovery of the bacteria. Bacteria form colonies in 2 days. Single colonies were usually multiplied on King's B agar medium. The bacterial isolates were named as BA1, BAL1, BP1, NP1. The DNA was isolated from different isolates and purified. The concentration of each DNA sample was measured by Nanodrop (Biotech instruments, USA). Here different housekeeping genes were targeted. A set of PCR primers that specifically detect the housekeeping genes such as inf B, gyr B, and rpo B of species *P. agglomerans, P. ananatis,* and *P. stewartii* with different base pairs was designed. A different set of primers was designed to detect the atp D gene, which is often found in all *Pantoea* species, and a standard set of primers was created to amplify the 16S rRNA region, which is present in all bacteria.

Table 1: List of PCR primers of the *Pantoea*-specific multiplex PCR scheme.

GENES	Base pair	Forward primer	Reverse primer
***P. agglomerans* (inf B)**	730	**5'-GATGACGARGCCATGCTGC-3'**	**5'-TGTCCGGCGTGCCGGCTG-3'**
***P. ananatis* (gyrB)**	423	**5'-GATGACGARGCCATGCTGC-3'**	**5'-GATCTTGCGGTATTCGCCAC-3'**
***P. stewartia* (rpoB)**	539	**5'-CACCGGTGAACTGATTATCG-3'**	**5'-GTCCTGAGGCATCAATGTGT-3'**
***Pantoea* sp(atpD)**	330	**5'-GAGGGTAACGACTTCTACCAC-3'**	**5'-CTGTACGGAGGTGATTGAAC-3'**
16sr RNA 27F and 1429R	1490	**5'-AGAGTTTGATCCTGGCTCAG-3'**	**5'-TACGGTTACCTTGTTACGACTT-3'**

Table 2: Composition of the multiplex polymerase chain reaction.

Step	Phase	Time	Temperature
1	Initial denaturation	3min	94°
2	Denaturation	30sec	94°
3	Annealing	30sec	58°
4	Extension	2min	72°
5	Cycling (steps 2-4)	35cycle	
6	Final extension	10min	72°

RESULTS AND DISCUSSION

On one side, many studies have shown that the use of Pantoea strains has resulted in significant enhancements in rice growth and productivity. This may be attributed to the advantageous impacts exhibited by these strains, as well as their ability to establish colonization within rice plants (Dutkiewicz *et al.,* 2016). Conversely, a multitude of publications have been published about the adverse effects of *Pantoea* on rice cultivation. This microorganism has been identified as an emerging pathogen in rice, leading to significant economic repercussions in terms of substantial financial losses (Lu *et al.,* 2021). The use of plant disease detection technologies is crucial in the field of epidemiological monitoring and in promoting the implementation of efficient management strategies (Martinelli *et al.,* 2015). But on the basis symptoms it is very difficult to distinguish between bacterial blight of rice caused by *Xoo* and *Pantoea* spp. So, the objective of this study was to develop diagnostic PCR primers specifically designed to target conserved housekeeping genes. PCR was run, and distinct bands were formed at 1.49kb for all the isolates, and it is because this region is highly conserved between different species of bacteria. The small subunit ribosomal RNA, which are encoded by the 16S rRNA gene, are necessary for the process of translation of mRNA to proteins which is common in all bacteria. Distinct bands were formed at 330 bp, where the atpD gene was amplified for the four isolates of *Pantoea* except *Xoo.* This at *pD* gene is commonly present in all the *Pantoea* spp., but it is absent in *Xoo.* The inf B gene, which is highly specific and only present in *P. agglomerans* and this region was amplified for the isolates BA1, BAL1 AND BP1, where distinct bands were formed at 730 bp. Further the VITEK-2 microbial identification system analysis and molecular characterization were confirmed the above three isolates as *Pantoea agglomerans.* Based on the analysis of whole-genome sequences, here a reliable multiplex PCR

protocol was followed given by Kini *et al.* (2021) that enables the specific detection of the these significant *Pantoea* spp. A number of plant diseases have been ascribed to a mere three out of the more than twenty-five species of *Pantoea*, specifically *P. agglomerans*, *P. ananatis, and P. stewartii.* Consequently, these three species can be regarded as the primary *Pantoea* spp. responsible for infecting plants. Various PCR methods have been employed for the purpose of diagnosing the condition, as documented by Coplin *et al*. (2002); Figueiredo and Paccola-Meirelles (2012); Ma *et al.* (2016). However, it should be noted that certain PCR methods have yielded amplicons that exhibit cross-reactivity with other species. Notably, most assays target only one *Pantoea* spp. or subspecies. The primary issue *P. stewartii subsp. stewartii*, which causes Stewart's bacterial wilt, may be identified by a number of techniques, but none of them simultaneously identify other *Pantoea* bacteria (Coplin *et al.,* 2002; Gehring *et al.,* 2014; Tambong, 2015; Thapa *et al.,* 2012; Xu *et al.,* 2010). Here to identify major plant-pathogenic *Pantoea* spp., a set of PCR primers that detect the bacterium species *P. agglomerans, P. ananatis, P. stewarti* and all *Pantoea* spp. was designed. A multiplex PCR scheme which can distinguish these species and also detects members of other *Pantoea* spp. was further developed.

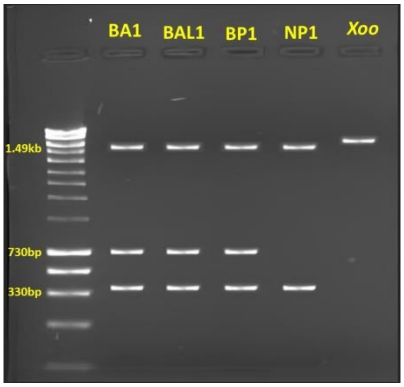

Fig. 1. Different isolates showing distinct bands with different base pairs.

Fig. 2. Panicle blight caused by *Pantoea* spp.

CONCLUSIONS

A new multiplex PCR scheme was developed to diagnose plant-pathogenic *Pantoea* spp. This tool enabled the efficient confirmation of the presence of *Pantoea* species mainly the *Pantoea agglomerans* in Odisha, which affecting rice production and productivity. When PCR was conducted, separate bands at 1.49 kb developed for each isolate. The atpD gene was amplified in distinct bands at 330 bp for all four *Pantoea* isolates except *Xoo*. *Pantoea agglomerans* isolates were amplified for the inf B gene, and separate bands for isolates BA1, BAL1, and BP1 appeared at 730 bp. In this manner, using mPCR, multiple sets of primers were utilized to concurrently amplify the bacterial DNA and separate bands were generated, differentiating *Pantoea* bacteria at the species level. This novel molecular diagnostic tool will aid in the correct identification of important *Pantoea* plant-pathogenic species. It will be particularly valuable for plant protection services and epidemiological monitoring of these major crop-threatening bacteria due to its reliability, specificity, sensitivity, and cost effectiveness.

FUTURE SCOPE

The disease has a sporadic occurrence in some of the major rice growing states of India and results in major loss to the concerned farmer. As it is very difficult to identify the causal organisms on their symptom's basis, such modern diagnostic tools help in identification of the actual causal agents of the disease.

Acknowledgement. The author is highly grateful to the Central Instrumentation Facility, Odisha University of Agriculture and Technology for providing the facilities required for this study.
Conflict of interest. None.

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How to cite this article: Biswajit Jena, A.K. Senapati and S.K. Behera (2023). Detection of *Pantoea* spp., an Emerging Pathogen of Rice through Multiplex PCR System. *Biological Forum – An International Journal*, *15*(1): 674-677.