Screening of antagonistic fluorescent Pseudomonads from rice rhizosphere for growth suppression of *Magnaporthe oryzae* and their molecular identification

**M Z I Zihad, A Sultana, F H Tumpa, S Chakraborty and M A R Khokon* 
**Department of Plant Pathology, Laboratory of Biocompounds, Bioformulation and Biosignaling, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

*E-mail: atiq.ppath@bau.edu.bd

**Abstract.** Isolation and identification of native fluorescent Pseudomonads from the rice rhizosphere were evaluated for their growth suppressing ability against blast disease of rice causing pathogen (*Magnaporthe oryzae*). Twenty strains of fluorescent Pseudomonads were isolated and identified initially based on their cultural and in vitro growth suppressing ability against *M. oryzae*. Among them BdPf4, BdPf8, BdPf9 and BdPf10 exhibited complete in vitro growth suppression (100%) against *M. oryzae* following dual culture in growth medium. Molecular identification of the antagonistic *Pseudomonas fluorescens* was done using 16S rDNA primers. Gel-electrophoresis of PCR products of all the isolates confirmed the fluorescent Pseudomonads showing band at 1500 bp. Phylogenetic analysis of the sequenced data revealed that BdPf8 (MN256392.1), BdPf9 (MN256393.1), BdPf10 (MN256394.1) have 100% homology with *Pseudomonas putida* (MT184822.1) from India, *Bacterium sp.* (MK823484.1) from China, *Pseudomonas sp.* (KY324900.1) from Brazil respectively. Seed priming by different strains of *P. fluorescens* significantly increased vigor index of rice seedlings. The highest vigor index (2274.8) and (2211.6) which was 184.42% and 176.53% higher over control treatment was found in BdPf16 (MN256399.1) and BdPf10 (MN256394.1) respectively. These results revealed the possibility of potential use of some of the strains of native *P. fluorescens* for management of blast disease of rice.

1. Introduction

Plant growth promoting rhizobacteria (PGPR) are capable of suppressing or preventing the phytopathogenic organisms that cause disease or damage in plant; therefore, PGPR are playing pivotal roles in emerging researches as biocontrol agent for managing different phytopathogens [1]. Among the biological control agents in PGPR group, fluorescent pseudomonads are attaining interest because of several mechanisms like competition for nutrients, production of bacterial metabolites such as iron chelating siderophores, hydrogen cyanide (HCN), antibiotics, extracellular lytic enzymes and induced systemic resistance [2]. Fluorescent pseudomonads are promising microbial agents that offer dual benefits of enhancing the crop growth and productivity while suppressing phytopathogens [3]. Moreover, seed bacterization with plant growth promoting bacteria is one of the commonly practiced methods of ameliorating plant growth and development. Seed priming with bacterial suspension extensively increases seed germination, shoot and root lengths and dry weight of seedlings [2]. Fluorescent pseudomonads are found to be producing significant amount of phytohormones like cytokinin [4], ACC deaminase [5], gibberellins [6], Auxin [5] which are involved in plant development at different stages like cell division, cell elongation, tissue differentiation and apical
dominance. The present research was undertaken to isolate and identify the antagonistic fluorescent Pseudomonads against *Magnaporthe oryzae* from the rice rhizosphere and their identification at molecular level.

2. Methodology

Fifty soil samples were collected from different Agro Ecological Zones (AEZs) of Bangladesh. Isolation of *P. fluorescens* was done according to the method developed by Meera and Balabaskar [7]. The growth suppression ability of *P. fluorescens* against *M. oryzae* was examined by dual culture technique (single line method) described in Jambhulkar *et al.* [8]. A 10 mm mycelial disc from 9 days old Corn Meal Agar (CMA) culture of *M. oryzae* was placed at one side of Petri dishes perpendicular to the bacterial streak and incubated at 25±2° C for 8-10 days and percent inhibition of pathogen growth was calculated by using the formula proposed by Vincent [9].

\[ I(\%) = \frac{(C-T)}{C} \times 100 \]

where, \( I = \) Inhibition, \( C = \) growth in control plate, \( T = \) growth in biocontrol agents inoculated plate

Total genomic DNA of purified growth-suppressing fluorescent pseudomonads were extracted by using the Wizard® Genomic DNA Purification Kit. For amplification of 16S rDNA, the used specific bacterial primers were: 16S FP- (5'- AGA GTT TGA TCM TGG CTC AG-3') and 16S RP- (5'- AAG GAG GTG ATC CAN CCR CA-3') [10]. The electrophoresis of PCR product gave band at the specific length. For further study, the 16S rDNA gene of all bacterial isolates were sequenced directly from PCR products in both orientations according to the standard protocols for the ABI A3500 genetic analyzer (Applied Biosystems, Foster city, CA, USA) with Big Dye® v1.1 and 3.1 Cycle Sequencing Kits. Vigor index of seedling were measured according to the formula proposed by Abdul-Baki and Anderson [11].

3. Results and Discussion

A total of 41 *Pseudomonas fluorescens* isolates were isolated from the rhizosphere of rice and screened for their growth suppressing ability against *M. oryzae*. In dual culture assay, twenty isolates showed mycelial growth suppression of *M. oryzae* at different level (Figure 1). Among twenty isolates BdPf-4, BdPf-8, BdPf-9 and BdPf-10 showed the highest (100%) growth suppressing effect against *M. oryzae* (Table 1). Isolates BdPf-7, BdPf-11, BdPf-12, BdPf-18 and BdPf-5 also inhibited mycelial growth 97.53%, 95.05%, 92.39%, 92.19% and 90.47% respectively (Table 1). In our previous study reported that complete growth suppression of *M. oryzae* can be found by some strains of *Pseudomonas* viz. Pf-6, Pf-7 and Pf-8 [12]. Subhalaksmi and Devi [13] found that *P. fluorescens* B24 showed the maximum mycelial growth inhibition (77.50%) of *M. oryzae* among the bacterial biocontrol agents against rice blast pathogen. They further reported that combined application of *P. fluorescens* and *Trichoderma* sp. (a fungal biocontrol agent) have significant influence on seed germination, root and shoot length in *in vitro* conditions and plant height was also increased by 5% in greenhouse and 12.02% in field trial with single application of *P. fluorescens* compared to control. In a similar kind of study, Suman [14] reported that, among thirty isolates of *P. fluorescens* from rice rhizospheric soil the organism DMP1 (an pseudomonad isolate collected from Mailaram location, Telangan district) showed highest *in vitro* growth inhibition (53.43%) of *Rhizoctonia solani*. To elucidate the mechanism of growth suppression by *P. fluorescens* Kumar *et al.* [15] reported that an inhibition zone is formed due to the secretion of antimicrobial metabolites during dual culture assay indicating the presence of biologically active metabolites which can diffuse through the agar medium and suppress the fungal growth. In order to know the variation among the growth suppressing isolates of *Pseudomonas fluorescens*, a phylogenetic tree was constructed with the most similar and homologous isolates registered in NCBI. Nucleotide analysis of the selected Pseudomonads in this study revealed that all the bacterial isolates showed maximum homology with different *Pseudomonas*
sp. and the strains of different places (Figure 1, Table 2, Figure 2). Out of 20 growth suppressing isolates, 19 isolates got NCBI accession and BdPf-20 could not register in the NCBI due to some error. The highest antagonism showing isolates BdPf-8, BdPf-9 and BdPf-10 showed 100% homology with *Pseudomonas putida* (MT184822.1) from India, *Bacterium* sp. (MK823484.1) from China, *Pseudomonas* sp. (KY324900.1) from Brazil respectively. The phylogenetic tree was constructed based on 16Sr DNA sequence of the native isolates of *P. flourescens* and the closest relatives previously registered in NCBI showed that all the isolates divided into seven clusters and in each cluster, isolates are closely related to *Pseudomonas* sp. from China, India, Nigeria and Mexico (Figure 3). The isolate BdPf-3 and BdPf-10 which were collected from Mymensingh district showed completely individual lineage in the phylogenetic tree. Seed bio-priming by seed inoculation with beneficial bacteria has become a newly acceptable substitute of harmful chemical for sustainable agriculture [16,17]. In this experiment, twenty growth suppressing bacterial isolates showed significant effect on shoot length and root length (Table 2). This finding is similar to the findings of Gholamalizadeh et al., [16] who studied rice associated bacterial ability to enhance seed germination and growth promotion of rice. They reported that MR$_5$ (*Bacillus vietnamensis*), MR$_2$ (*Bacillus idriensis M50*), OR$_4$ (*Alcaligenes faecalis*), DEp$_8$ (*Alcaligenes faecalis*) and FE$_1$ (*Stenotrophomonas maltophilia*) treated seeds had the same germination rate. Mia et al.[18] also found similar results that the treatments did not yield any differences in emergence, at 48 to 96 h after inoculation of seeds with the strains of *Rhizobium* sp. (UPMR 1006) while *Rhizobium* sp. (UPMR 1102) showed higher percent emergence compared to other *Rhizobium* sp. Vigor index reflects the seedlings health, establishment and the state of final productivity of the plant [19].

| Sl. No. | Name of Isolates | Mycelial growth 9 DAI (mm) | % Growth inhibition |
|--------|------------------|---------------------------|-------------------|
| 1      | BdPf-1           | 11.11 e                   | 80.95             |
| 2      | BdPf-2           | 18.11 c                   | 68.95             |
| 3      | BdPf-3           | 17.11 cd                  | 70.67             |
| 4      | BdPf-4           | 0.00 j                    | 100               |
| 5      | BdPf-5           | 5.56 fg                   | 90.47             |
| 6      | BdPf-6           | 12.45 e                   | 78.66             |
| 7      | BdPf-7           | 1.44 ij                   | 97.53             |
| 8      | BdPf-8           | 0.00 j                    | 100               |
| 9      | BdPf-9           | 0.00 j                    | 100               |
| 10     | BdPf-10          | 0.00 j                    | 100               |
| 11     | BdPf-11          | 2.89 hi                   | 95.05             |
| 12     | BdPf-12          | 4.44 gh                   | 92.39             |
| 13     | BdPf-13          | 28.66 b                   | 51.05             |
| 14     | BdPf-14          | 11.33 e                   | 80.57             |
| 15     | BdPf-15          | 11.22 e                   | 80.76             |
| 16     | BdPf-16          | 7.33 f                    | 87.43             |
| 17     | BdPf-17          | 13.22 e                   | 77.33             |
| 18     | BdPf-18          | 4.55 gh                   | 92.19             |
| 19     | BdPf-19          | 15.77 d                   | 72.96             |
| 20     | BdPf-20          | 11.11 e                   | 80.95             |
| 21     | Control          | 58.33 a                   | .....             |

CD (0.05) 2.216  
CV% 12.31

* DAI= Days After Incubation * CV= Coefficient of Variation, Critical difference (CD)
Figure 1. Growth suppression of *M. oryzae* by selected fluorescent pseudomonads in dual culture assay (single line method).
Table 2. Efficacy of seed bio-priming by fluorescent pseudomonads isolates on seed germination, shoot length, root length and vigor index.

| Isolates     | Germination % | Shoot length (cm) | Root length (cm) | Vigor index |
|--------------|---------------|-------------------|------------------|-------------|
| BdPf-1       | 93.33         | 8.90 b-d          | 6.58 b-d         | 1444.80 b   |
| BdPf-2       | 100.00        | 7.46 c-i          | 4.63 e-i         | 1210.00 b-f |
| BdPf-3       | 93.33         | 6.80 e-j          | 3.70 hi          | 981.43 f-h  |
| BdPf-4       | 93.33         | 8.37 c-f          | 5.42 c-g         | 1289.30 b-e |
| BdPf-5       | 86.66         | 8.02 c-h          | 7.41 b           | 1319.70 b-d |
| BdPf-6       | 96.66         | 7.46 c-i          | 4.58 f-i         | 1160.13 c-f |
| BdPf-7       | 96.66         | 6.53 h-j          | 4.37 g-i         | 1055.03 e-g |
| BdPf-8       | 90.00         | 9.02 b-d          | 6.69bc           | 1411.83 b   |
| BdPf-9       | 90.00         | 6.96 e-j          | 4.44 g-i         | 1026.60 f-h |
| BdPf-10      | 100.00        | 13.01 a           | 9.10 a           | 2211.66 a   |
| BdPf-11      | 93.33         | 6.64 f-j          | 4.53 f-i         | 1044.23 e-h |
| BdPf-12      | 93.33         | 10.29 b           | 4.80 e-i         | 1423.43 b   |
| BdPf-13      | 93.33         | 6.57 g-j          | 3.73 hi          | 963.56 f-h  |
| BdPf-14      | 90.00         | 7.77 c-h          | 4.59 f-i         | 1113.00 d-g |
| BdPf-15      | 90.00         | 9.25 bc           | 5.96 b-f         | 1369.80 bc  |
| BdPf-16      | 96.66         | 13.83 a           | 9.73 a           | 2274.80 a   |
| BdPf-17      | 96.66         | 8.50 b-e          | 5.12 d-h         | 1316.73 b-d |
| BdPf-18      | 90.00         | 7.37 d-i          | 4.74 e-i         | 1090.20 d-g |
| BdPf-19      | 93.33         | 5.89 ij           | 3.45 i           | 874.500 gh  |
| BdPf-20      | 90.00         | 8.36 c-g          | 6.14 b-e         | 1305.90 b-d |
| Control      | 90.00         | 5.40 j            | 3.48 i           | 799.80 h    |

CD (0.05)  Not significant  1.802  1.518  248.739

*CV%  5.239  13.334  17.068  11.873

* CV= Coefficient of Variation, Critical Difference (CD)
Figure 3. Phylogenetic tree was constructed based on neighbor-joining method using 16S rDNA profiles of the native isolates of the *Pseudomonas flourescens* and their closest relatives found in the NCBI.
Results obtained in this study showed that the seedling lengths were significantly increased by bacterial inoculations by the isolates BdPf-16 (2274.8) and BdPf-10 (2211.666) resulting increasing higher vigor index. The isolates BdPf-16 and BdPf-10 were also able to increase vigor index of rice seedlings. Some researchers reported that for effective plant growth promoting bacteria, they must be able to establish themselves and colonize seed to increase seedling length as well as vigor index of seed [11].

This study concluded that the isolated fluorescent pseudomonads were capable of suppressing M. oryzae in laboratory condition. It is very important to test the efficacy of these isolates under field condition. The findings of the present study expose new area to investigate on antimicrobial activities. Besides, it is also important to study growth promotion activity, colonizing ability on leaf and root surface at field level before commercialization as bio-control agent. An effective bio-control agent can be a sustainable and environmentally safe way to manage rice blast disease.

Acknowledgement
The study was jointly funded by the Ministry of Science and Technology, The peoples’ Republic of Bangladesh and The Ministry of Education (Project no. 2017/195/MoE).

References
[1] Nihorembere V, Ongena M and Smargiass M 2011 Beneficial effect of the rhizosphere microbial community for plant growth and health Biot. Agron. Soc. Environ. 15 327–37
[2] Vanloon L C, Bakker P A H M and Pieterse C M J 1998 Systemic resistance induced by rhizosphere bacteria Annl. Rev. Phytopathol. 36 453–83
[3] Raaijmakers J M and Weller D M 1998 Natural plant protection by 2,4-diacetylphloroglucinol producing Pseudomonas spp. in take-all decline soils Mol. Pl. Microb Int. 11 144–52
[4] Vessey K J 2003 Plant growth promoting rhizobacteria as biofertilizers Plant Soil 255 571–86
[5] RavindraNaik P, Raman G, Badri Narayanan K and Sakthivel N 2008 Assessment of genetic and functional diversity of phosphate solubilizing fluorescent pseudomonads isolated from rhizospheric soil BMC Microbiol. 8 230
[6] Gutierrez-Manero F J, Ramos B, Probanza A, Mehouachi Jand Talon M 2001 The plant growth promoting rhizobacteria Bacillus pumilus and Bacillus licheniformis produce high amounts of physiologically active gibberellins Physiol. Plant. 111 206–11
[7] Meera T and Balabaskar P 2012 Isolation and characterization of Pseudomonas fluorescens from rice fields Int. J. Food Agri. Vet. Sci. 2 113–20
[8] Jambhulkar P P, Sharma P, Manokaran R, Lakshman D K, Rokadia P and Jambhulkar N 2018 Assessing synergism of combined applications of Trichoderma harzianum and Pseudomonas fluorescens to control blast and bacterial leaf blight of rice Euro. J. Pl. Path. 512 747–57
[9] Vincent J M 1947 Distortion of fungal hyphae in the presence of certain inhibitors. Nature 159 850
[10] Hauben L, Vauterin L, Swings J and Moore E R B 1997 Comparison of 16s ribosomal DNA sequences of all Xanthomonas species Int. J. System. Bacteriol. 47 328–35
[11] Abdul-Baki A A and Anderson J D 1973 Vigor determination in soybean seed by multiple criteria Crop Sci. 13 630–33
[12] Chakraborty S, Tumpa F H and Khokon M A R 2020 Development of formulation of fluorescent pseudomonads and its evaluation on bio-management of blast of rice Arch. Phytopathol. Pl. Prot. 1–22
[13] Subhalakshmi T and Devi I S 2017 Blast of rice in Manipur and its biocontrol by Pseudomonas fluorescens and Trichoderma sp. Int. J. Curr. Microbiol. Appl. Sci. 6 1619–34
[14] Suman B 2015 Isolation and characterization of Pseudomonas fluorescens from rice fields for the biological control of Rhizoctonia solani causing sheath blight in rice. M.S. Thesis, department of agricultural microbiology, Professor Jayashankartelangana state agricultural
university.

[15] Kumar S, Stecher G and Tamura K 2016 MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets Mol. Biol. Evol. 33 1870–74

[16] Gholamalizadeh R, Khodakaramian G and Ebadi A A 2017 Assessment of rice associated bacterial ability to enhance rice seed germination and rice growth promotion Braz. Arch. Biol. Tech. 60 1678–24

[17] Rahman N M, Tumpa F H, Islam A K M S and Khokon M A R 2020 Bio-priming of cucurbits and okra seeds with culture filtrates of Trichoderma harzianum for the controlling of seed-borne fungi J. Bang. Agri. Univ. 18 12–16

[18] Mia M A B, Shamsuddin Z H and Mahmood M 2012 Effects of rhizobia and plant growth promoting bacteria inoculation on germination and seedling vigour of low land rice Afr. J. Biotechnol. 11 3758–65

[19] Mcdonald M B and Copeland L O 1997 Seed production: principles and practices. Chapman & hall, London. pp. 719