ISSN 2250-3153

Isolation of Unique Gram Positive Rod from Diseased Rice Leaves

Samya Sen, Sunanda Mandal, Anirban Mullick, Sounak Kundu, Debanjan Goswamy, Swati Mukerjee, Mithun Das, Arup Kumar Mitra

Post-Graduate Department Of Microbiology, St. Xavier's College, 30, Mother Teresa Sarani, Kolkata-700016

Abstract- Rice (*Oryza sativa*) is an important food crop of India and the world, is frequently attacked by pathogens like bacteria, fungi, viruses, pests and weeds. These diseases reduce both quantity and quality of plant products. In this present investigation unique Gram positive pathogenic bacteria, *Bacillus cereus* (as identified by NCBI BLAST)along with fungal spores of *Bipolaris sp* pathogens shared the same leaves of Lal Swarna variety of rice and showed dark brown spots with yellow halo on leaves. Here, *Bacillus cereus* which maximum sensitive to Sulbactam and least to Ceftadime was not a pathogen of rice before and it is first time observed as a pathogen of rice and at the same time it shows antagonism with *Bipolaris sp.* growth. This investigation also shown this mixed infection resulted in less vigorous infection of *Bipolaris* to this rice plant and which can be utilized in biological control in place of pesticides.

Index Terms- Bacillus cereus, Koch's Postulate, Lal Swarna, 16s rRNA Sequencing.

I. INTRODUCTION

Rice *(Oryza sativa*) is a staple crop all over the world belongs to the family of Gramineae (Poaceae).The genus *Oryza* contains 25 recognized species, of which 23 are wild species and two, *Oryza sativa* and *Oryza glaberrima* are cultivated (Olga F. Linares 2002). *Oryza sativa* is the most widely grown of the two cultivated species. Within India, rice occupies one-quarter of the total cropped area, contributes about 40 to 43 percent of total food grain production and continues to play a vital role in the national food and livelihood security system. In India ,West Bengal is the highest rice producing state while Tamil Nadu has first place in productivity. But, it has been estimated that the world wide annual yield loss and decrease in nutritive values are mainly due to diseases. Rice diseases are mainly caused by fungi, bacteria ,viruses or insects . So it is important to choose different varieties of rice and investigate the disease and find out the possible way to combat it because diseases are considered major constraints in rice production and nutrient intake to people. For this purpose diseased rice plants locally known as Lal Swarna variety of rice was collected from Katwa(23.6411° N, 88.1347° E) in Burdwan district, West Bengal, India and the investigation was undertaken with the following objectives:

- **To indentify a particular variety showing high rate of disease infection.**
- **To isolate the obtained pathogenic forms in their pure culture.**
- **To determine their effect of interaction between these pathogens.**
- **To suggest eco-friendly ways of treating the disease.**

Thus, via a series of experiments carried out, we try to draw up a conclusion of how bacteria and fungi interact to outdo each other and if possible, finding out a potential bio-pesticide or bio-fungicide for disease control because the use of chemical fungicides or pesticides are damaging the ecosystem at large and little effect in curing any disease.

II. MATERIALS AND METHOD

A) ISOLATION AND STAINING:

Infected leaves with specific lesions were selected and washed in sterile water to remove mud and dust and cut into pieces. These pieces were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution and transferred aseptically to PotatoDextroseAgar slant containing streptomycin and Nutrient Agar slant for isolating fungi and bacteria and incubated for isolating pure colonies.The pure colonies were chosen and maked as Sample B and sample F for bacterial and fungal culture respectively.

B) KOCH'SPOSTULATE

To confirm the pathogenicity of both bacteria and fungi by Koch postulate, healthy leaves of same rice plant were inoculated with suspension of Sample B and F and incubated at room temperature .

C) SLIDE BIOASSAY

In this investigation the slide bioassay was done to study of growth and development of these microorganisms in presence of four different nutrition conditions for 24 hours.

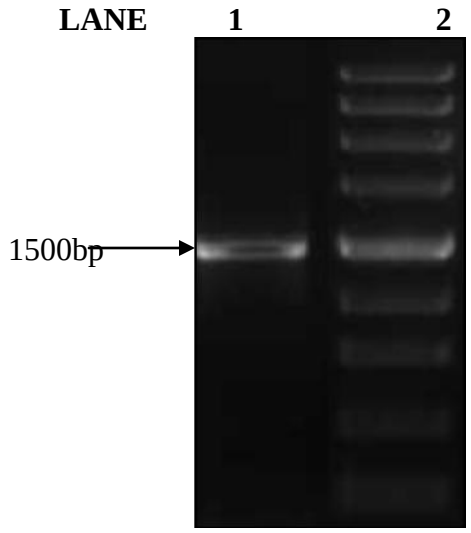
D) ANTIBIOTIC SENSITIVITY TEST

The antibiotic sensitivity test was carried on for Sample B to check the resistance and sensitive antibiotics. This assay was done by disc diffusion test by following Kirby-Bauer method with Ampicilin, Sulbactam Nitrofurantoin, Ceftadime, Linezolid, Ciprofloxacin, Streptomycin and Tetracyclin.

E) IDENTIICATION OF SAMPLE B BY 16S rDNA

Bacterial 16S rDNA sequences are attractive targets for developing identification methods because they represent conserved regions in all bacteria and species having 70% or greater DNA similarity usually have more than 97% sequence

identity (Stackebrandt and Goebel, 1994).Bacterial identification based on % similarity of 16S rDNA has been using PCR technique, DNA sequencing and similarity analysis of rRNA genes. A direct comparison of 16S rDNA sequence is probably the most powerful tool for the identification of many bacteria (Stackebrandt and Goodfellow, 1991). 16S rDNA was amplified and sequenced using oligonucleotide primers complementary to highly conserved regions of bacterial rRNA gene. For identification ,DNA was isolated from the slant culture of Sample B. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. (Figure 1).The PCR amplicon was purified to remove contaminants.Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer and consensus sequence was generated by Aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database (Marchler Bauer et al., 2000; Pruitt et al., 2005). Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W.



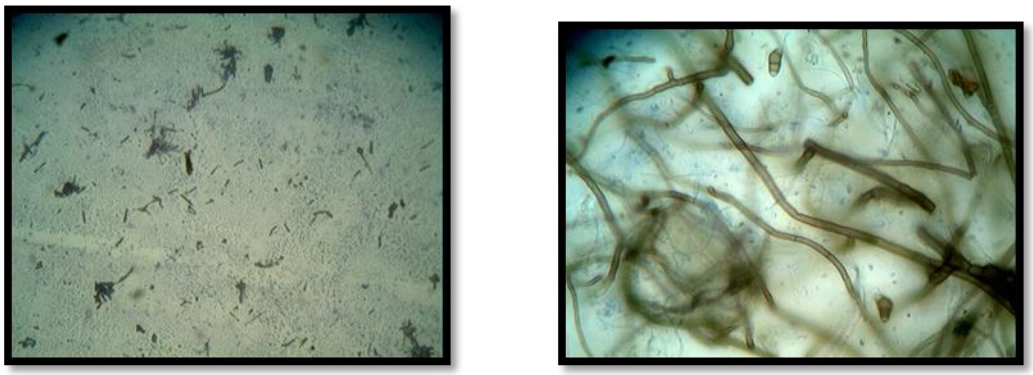
Lane 1: 16S rDNA amplicon band ,Lane 2: DNA marker

FIGURE 1: Gel Image Of 16s rDNA Amplicon (Sample: B)

III. RESULTS

India is an important rice producer of the world. According to the India's Agriculture Ministry the country had harvested about 103.41 million tons of rice in the 2011-12 crop year (source: PTI 2012, The Economic Times) in which rice output from West Bengal was about 1.51 core tons in 2011-12 (source: PTI 2012, The Times of India). But, the infections act as a devastation for rice plants in India and West Bengal. In the country the rice plants are mainly attacked by bacterial pathogen like Gram negative *Xanthomonas oryzae* pv. oryzae (Ray, P.R.et al, 1970) causing Bacterial Leaf Blight with yield loss of 6% to 60%. *Xanthomonas oryzae* pv.oryzicola (David O., et. al 2006) also causes Bacterial Leaf Streak with losses as high as 32.3% in 1000-grain weight. In India they are also attacked by numerous fungal pathogens like *Bipolaris oryzae* (Julie Flood 2010) causing Brown Spot and results in 14-41% losses in high yielding varieties. *Ustilaginoidea virens* (Dodan DS, Singh R. 1996; Biswas A.2001) causing False Smut in India and resulting in yield loss of 7-75%. *Pyricularia oryzae* (Neergaard et.al 1970) produces Rice Blast. In India, it results in about 0.8% of their total yield loss. But from various observations it was found out *Bipolaris sp*. is one of the severe pathogen of different varieties of rice in West Bengal. The disease Brown Spot was considered to be the major factor contributing to the "Great Bengal Famine" in 1942(Julie Flood 2010) resulted in yield losses of 50% to 90% and the death of two million people. So, *Bipolaris* infection is one of the major concern for the rice producer in West Bengal. Even *Bipolaris* is a major disease causing pathogen of Lal Swarna variety of rice India. In our investigation, staining of pure colonies of Sample B and F (TABLE I) from diseased leaves showed the bacteria to be Gram Positive *Bacillus sp.* (FIGURE 2a)and fungus to be *Bipolaris sp*. (FIGURE2b). Next,the reinoculation of isolated pathogens (Sample B and Sample F) from diseased leaves to healthy leaves showed the growth of these pathogens with their disease symptoms as seen in diseased leaves of rice plant .These symptoms and percentage of infection (Table II) indicate that both the organisms are pathogenic to this rice leaf. The observation (TABLE III), showed the presence of sugar increases the germination of conidia and also the remarkably increased the germ tube length of the fungi. It further showed that fungicide reduce the germ tube length by 16.24% and the pesticide reduce the germination of germ tube by only 1.36% than in presence of sugar solution.Whereas infection of *Bacillus cereus* reduced the germ tube germination by 8.25%. So there is definite antagonism between the two organisms(SampleB and Sample F), which can be utilized in biological control. Thus, it is evident from this investigation that the use of pesticide is not necessary in this form of infection because of the antagonism existing between the two organisms. Thus, the in-vitro slide assay has also shown that use of chemical Fungicide (Saaf) and Pesticide (Thiodan) are of little help to suppress these pathogens because the growth of fungal pathogen was not fully suppressed and it might cause some health hazards. Another important part of the investigation was to find out the sensitive antibiotics for the pathogenic Gram positive bacteria (Sample B) and so antibiotic sensitivity test was performed because bacterial diseases can be easily cured by applying sensitive antibiotics. The result of disc diffusion test (Table IV) showed that the Sample B was resistant to those antibiotics for whom the zone of diameter were less than 20mm and sensitive to those antibiotics for whom the zone of diameter were more than 20mm i.e . Sample B is maximum sensitive to Sulbactam and least to Ceftadime. (Clinical And Laboratory Standards Institute Performance Standards for Antimicrobial Disk Susceptibility Tests, Tenth edition 2008. Microbiology: A laboratory Manual :International Ninth Edition. Cappuccino and Sherman 2011, Page No. 293). Lastly,16s rRNA characterization showed (SAMPLE B) to be *Bacillus cereus*(TABLES V,VI and FIGURE

5). In the distribution of 283 blast hits on the query sequence of 1439bp matched the alignment scores ≥200. Sequence producing significant alignments by BLAST closely matched to *Bacillus cereus*, and different strains of *Bacillus sp*. were also found to be close to this species. Expect value (E value) of all these strains is 0.0 which depicts that all the strains are homolog to *Bacillus cereus* . This pathogen is more common in the production of toxins in the rice product and in turn induces food infection rather than pathogen to rice crop. Moreover, finding of sensitive antibiotics by the disc diffusion test may help us to suppress diseases caused by sole infection of this new pathogenic strain of *Bacillus cereus* of rice plant in future and improve the quality and quantity of rice production in West Bengal and India.



a) Bacillus sp. (observed at 100X) b) *Bipolaris* Mycelia And Spores (Observed At 45X)

Figures 2 Microscopic view of sample B and F

Table I: Result of isolation and staining

Part Of infection	Microscopic features	Sample	Microorganism (Probable)
Leaf	Gram positive rods	Sample B	*Bacillus sp*
	1. Presence of arthrospore (round, all spores joined to each other, spores intercalary, hyaline, thick walled) 2. Presence of conidia (mature-sickle shaped and colour brownish black)	Sample F	*Bipolaris sp*

Table II: Result of Koch's Postulate

Name of pathogen	Number of leaves inoculated	Number of leaves infected	Appearance & nature of spots	Percentage of infection
Sample B	89	48	Apex of the leaf accompanied with the browning reaction due to polyphenol oxidase reaction observed .	54.93
Sample F	78	59	Irregular brown spot without any halo observed after 2 weeks.	76.64

Table III: Result of slide Bio- Assay

Name of slides	Suspension of microorganism	Solution		Percentage of Germination	Length of germ tube (µm)
CONTROL 1	Sample F suspension	Sterile Water		14.55	16.65
CONTROL 2	Sample F suspension	Sugar (2%)		20.83	35.75
SLIDE 1	Sample F suspension + Sample B suspension	Sterile Water		11.62	15.54
SLIDE 2	Sample F suspension + Sample B suspension	Sugar (2%)	Incubation for 48 hours	12.58	18.32
SLIDE 3	Sample F suspension	Fungicide-Saaf (5mg/100ml)		4.59	2.67
SLIDE4	Sample F suspension	Pesticide Thiodan (0.6ml/100ml)		19.47	4.45

Table IV: Result of antibiotic sensitivity test

Name of antibiotic	Concentration (µg)	Diameter (mm)	Mean (mm)
Ceftadime	30	0	0
Linezolid	30	34 36	35
Ciprofloxacin	5	23.3 24.6	23.95
Nitrofurantoin	300	15.6 15.6	15.6
Streptomycin	10	15 16.5	15.75
Tetracyclin	30	22 21.6	21.8
Ampicilin	10	10 10	10
Sulbactam	105	30 30	30

TABLE V : Consensus sequence of SAMPLE B (1439 bp)

TGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACC
GCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAA
CGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGC
TTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAG
AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAA
GCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACT
TGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTG
GGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAAT
CATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGC
TAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGG
ATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAA
GTCGGTGGGGTAACCTTTTGGAGCCAGCCGCC

Table VI: Sequence producing significant alignments (source:http://blast.ncbi.nlm.nih.gov/)

Sequences producing significant alignments:						
Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
AP007209.1	*Bacillus cereus* NC7401 genomic DNA, complete genome	2658	3.703e+04	100%	0.0	100%
JN187086.1	*Bacillus cereus* strain YC-16 16S ribosomal RNA gene, partial sequence	2658	2658	100%	0.0	100%
JF506009.1	*Bacillus anthracis* strain KNUC9075 16S ribosomal RNA gene, partial sequence	2658	2658	100%	0.0	100%
JF833090.1	*Bacillus cereus* strain Js16 16S ribosomal RNA gene, partial sequence	2658	2658	100%	0.0	100%
GU982920.1	*Bacillus cereus* strain GXBC-1 16S ribosomal RNA gene, partial sequence	2658	2658	100%	0.0	100%
FN663625.1	*Bacillus* sp. OU-A3 16S rRNA gene, strain OU-A3	2658	2658	100%	0.0	100%
AB116124.1	*Bacillus anthracis* gene for 16S ribosomal RNA, partial sequence, strain: S51	2654	2654	100%	0.0	99%
AY138332.1	*Bacillus anthracis* strain 2000032707 16S ribosomal RNA gene, partial sequence	2649	2649	100%	0.0	99%
AB295053.1	*Bacillus thuringiensis* gene for 16S rRNA, strain: NK2	2652	2652	100%	0.0	99%

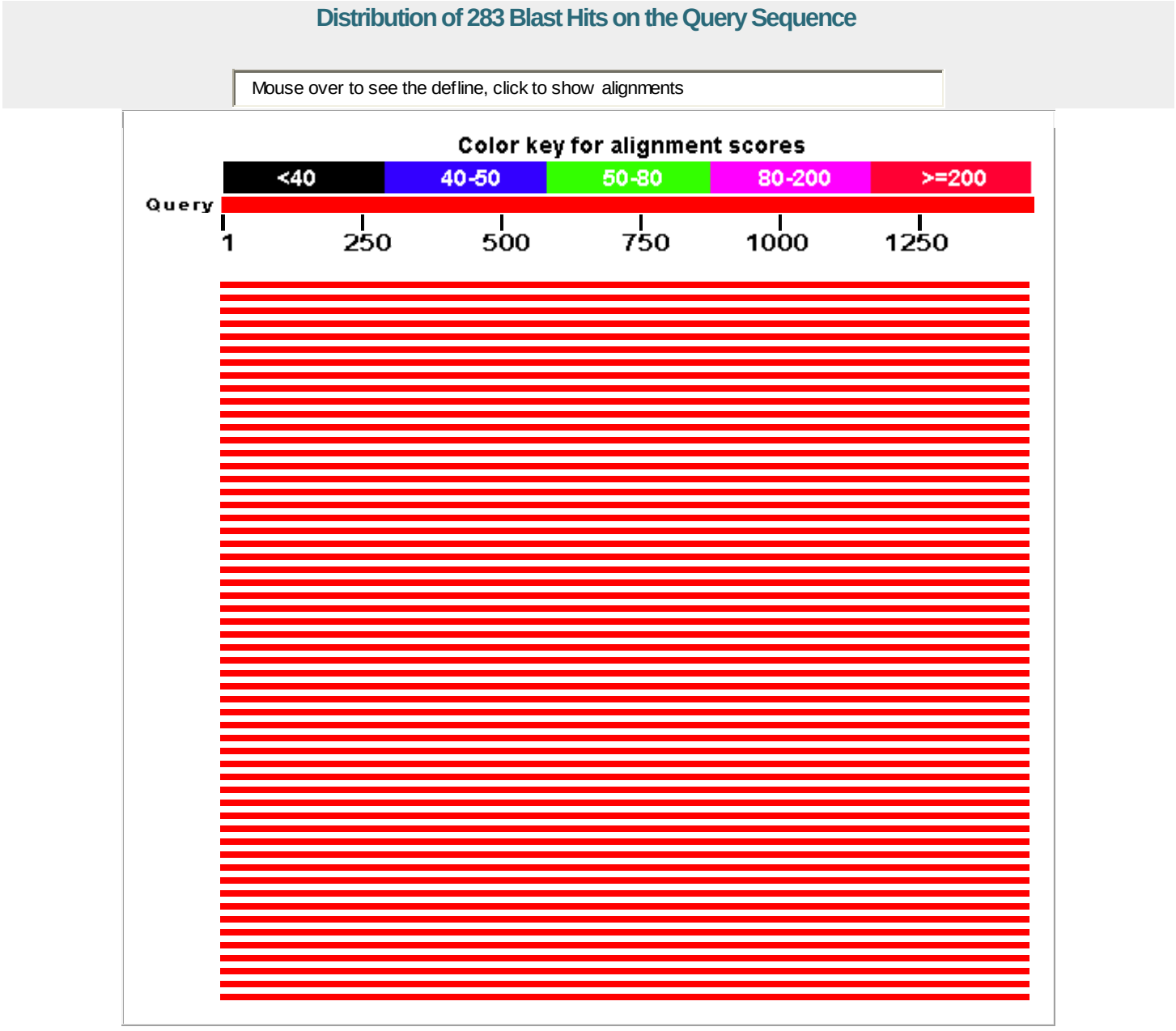


Figure 5: Distribution Of 283 Blast Hits On The Query Sequence
(source: BLAST DATA: ALIGNMENT VIEW USING NCBI GENBANK;http://blast.ncbi.nlm.nih.gov/)
Information about other close homologs for the microbe can be found from the Alignment View table.

IV. CONCLUSION

This finding is unique because Bacillus cereus was not a pathogen of rice before and it is first time reported as a pathogen of rice and at the same time it shows antagonism with Bipolaris sp. very strangely. From this investigation it was found out that when there is a mixed infection of Bipolaris sp. along with Bacillus cereus (as identified by 16S rDNA analysis), the infection caused by Bipolaris was of less intensity. So this how joint infection can reduce vigorous infection of Bipolaris sp. and at the same time can protect the severe loss of rice crop.

ACKNOWLEDGEMENT

We express our sincere thanks to Rev.Fr.Dr.J.Felix.Raj, Principal of St. Xavier's College, Kolkata, India for his support to carry out these experiments. We are grateful to Xcelris Labs Ltd., Ahmedabad, India for the 16S rRNA sequencing and all the laboratory attendants for their support in this work..

REFERENCES

[1] Biswas A.Changing trends of rice diseases in West Bengal, India. Journal of Mycopathological Research Vol. 38 No. 1,Record Number20003036449. 2000 ,33-36.

[2] Biswas A. False smut disease of rice: a review. Environ. Biol. 19: 2001,67-83.

[3] Cappuccino and Sherman Microbiology: A laboratory Manual : International Pearson Education .Ninth Edition. 2011, 293

[4] David O., NinoLIiu, Pamela, C. Ronald and Adam J. BogdanoveXanthomonas oryzae pathovars: model pathogens of a model crop. Molecular Plant Pathology. 2006 ,303–324.

[5] Dodan DS, Singh R.. False smut of rice: present status. Agric. Res. 17: 1996 ,227-240.

[6] Drobniewski, F. A. Bacillus cereus and related species. Clin. Microbiol. Rev. 63: . 1993,324-338

[7] E. Stackebrandt and B. M. Goebel .Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology . JSEM vol. 44 no. 4. 1994, 846-849.

[8] E. Stackebrandt and M. Goodfellow . Nucleic Acid Techniques in Bacterial Systematics (Modern Microbiological Methods). John Wiley & Sons. ,Chichester — New York — Brisbane — Toronto — Singapore. 1991,ISBN: 0–471–92906–9.

[9] Julie Flood. The importance of plant health to food security. Springer Science+Business Media B.V. & International Society for Plant Pathology Food Sec., 2: 2010, 215–231

[10] Marchler-Bauer, A., Panchenko, A. R., Shoemaker, B. A., Thissen, P.A., Geer, L. Y. and Bryant, S. H. CDD. A database of conserved

[11] domain aligments with links to domain three-dimensional structure.Nucleic Acids Res. 30: 2000, 281-283.

[12] Motlagh, M.R. and B. Kaviani. Characterization of new Bipolaris spp.: The causal agent of rice brown spot disease in the North of Iran. Int. J. Agri. Biol. 10: 2008 , 638–42.

[13] Neergaard, Paul, Lambat, A. K. and Mathur, S. B. Seed Health testing of rice III. Testing procedures for detection of Pyricularia oryzae Cav. Proc. int. Seed Test. Ass.,35. 1970, 157-163.

[14] Olga F. Linares African rice (Oryza glaberrima): History and future potential. pnas. 252604599P NAS vol. 99 no. 25: 2002 ,16360-16365.

[15] Pruitt, K. D., Tatusova, T. and Maglott, D. R . NCBI ref. seq. a curaled non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res. 33: 2005, D501-D504.

[16] PTI . The Economic Times, 2012 http://articles.economictimes.indiatimes.com.

[17] PTI . The Times Of India,2012 http://articles.timesofindia.indiatimes.com.

[18] Ray, P.R.; Sengupta, T.K. A study on the extent of loss in yield in rice due to bacterial blight. Indian Phytopathology 23: 1070, 713-714.

[19] Weller, R. and D.M. Ward. Selective recovery of 16S rRNA sequences fromnational microbial communities in the form of cDNA. Appl. Environ. Microbiol. 55: 1989,1818-1822.

AUTHORS

First Author – Samya Sen .B.Sc , Post Graduate student,St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016, India and email : samya.sen1990@gmail.com
Second Author – Sunanda Mandal. B.Sc , Post Graduate student, St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016 India and email : mandal.sunanda89@gmail.com
Third Author – Anirban Mullick. B.Sc , Post Graduate student ,St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016, India and email :mullick4@gmail.com
Fourth Author – Sounak Kundu. B.Sc , Post Graduate student ,St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016 India and email : sounak.dx@gmail.com
Fifth Author – Debanjan Goswamy .B.Sc , Post Graduate student ,St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016 India and email : debanjango@gmail.com
Sixth Author – S wati Mukerjee. B.Sc , Post Graduate student ,St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016 India and email : mukherjeemicroswati89@gmail.com
Seventh Author – Mithun Das.B.Sc , Post Graduate student ,St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016 India and email : mithunsoft@gmail.com

Correspondence Author – Dr.Arup Kumar Mitra, M.Sc. Ph.D(Cal),Head Department of Microbiology St. Xavier's College 30, Mother Teresa Sarani, Kolkata-700016,e-mail: drakmitra01@hotmail.com and Contact number:+919831041051,+913324795169(Resi),+913322551276/ +913322551285(College)