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ANTAGONISTIC RELATIONSHIP BETWEEN *BACILLUS CEREUS* AND *BIPOLARIS SP* IN THE LEAF SPOT DISEASE OF *BASELLA ALBA:* A NOVEL FINDING

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ABSTRACT

***AIM OF STUDY**: To investigate the phyllospheric interaction behind the leaf spot disease of Basella alba, a globally used vegetable as well as medicinal plant, better known as Malabar spinach. **MATERIALS AND METHODS:** Spores of bacteria and fungus were isolated from the infected zone and applied to fresh leaves to validate Koch's postulates. Slide bioassay test was performed to investigate the interaction between pathogenic fungus and bacterial species. Antibiotic assay and fungicide assay were performed for bacterial and fungal species respectively. Also 16S rDNA study was done to identify the bacterial species. **RESULT:** Bacillus cereus, the bacterial species (Identified by NCBI blast) was found to show antagonistic relationship with the pathogenic fungal species Bipolaris sp. (characterized by spore study). **CONCLUSION:** Bipolaris sp. was previously reported for its contribution to leaf spot disease but the role of Bacillus cereus (the bacteria was found to cause 35 % infection in the leaf) in causing the same is being reported for the first time. Moreover, Bacillus cereus suppresses the growth of the potential plant pathogenic fungi Bipolaris spp.(according to slide bioassay test) showing a novel approach in biological control of the leaf spot disease in Basella alba.*

KEY WORDS

Bacillus cereus, Bipolaris sp , Basella alba, Koch's postulates,16S rDNA

INTRODUCTION

Malabar spinach,(Basella alba) today is cultivated as a food plant throughout the warmer regions of the world [1]. *Basella alba* (Basellaceae family) or Malabar Spinach is a perennial vine native to tropical Asia **(http:// www.worldcrops.org/crops/Malabar-Spinach.cfm),** where it is widely used as a leaf vegetable. There are 2 main species of Malabar spinach: Basella alba, which has green stem and thick fleshy leaves, and *Basella rubra* which has red stem.

Typical of leaf vegetables, B .alba is high in Vitamin A, Vitamin C, iron and Calcium. The succulent mucilage is particularly rich source of soluble fiber and has antioxidant properties. Leaf extract also contains protein, fat, Vitamin E, Vitamin K, Riboflavin, niacin, Vitamin B9 and Magnesium [2].

In Ayurveda, the leaves or aerial parts and stem of B.alba have been used for the treatment of hemorrhoids, sexual weakness, anemia, constipation, ulcers and as a diuretic, laxative, anti-cancer such as melanoma, leukemia. It's use has also been discovered as anticonvulsant, rubefacient, asperient, demulcent, anti-inflammatory, androgenic, antipyretic and for catarrhl afflictions [2]. Spoilage of green leafy vegetable is due to the activity of microorganisms, the condition favorable for their proliferation being moisture and warmth [3]. The spinach has high moisture content ranging from 90.50% in the green variety (*Basella alba*) and 90.00% in the purple variety (*Basella rubra*)[4] which contribute in microbial infection thereby leading to spoilage and loss of quality. The plant is of great

significance as a dietary medicine and can serve as a potential source of new drugs.Hence, a proper study of the microorganisms pathogenic to it is important. A study was conducted wherein, Bacillus cereus, which is used as a biological control for keeping certain plant pathogens under check was found to be the causal organism of leaf spot of B.alba. For this purpose diseased B.alba leaves locally known as pui shak were collected from Kolkata (Kudghat), West Bengal and the investigation was undertaken with the following

OBJECTIVES:

1. To isolate the obtained phyllospheric microbes in pure culture.
2. To establish their pathogenic nature.
3. To determine the effect of interaction between these pathogenic organisms.
4. To evolve a control method for these pathogenic organisms, preferably by using their interactive effects.

MATERIALS AND METHODS

A) Isolation of Potential Plant pathogen

Infected leaves were collected from a local *Basella alba* plant and the identical grey spots were cut off from the leaf with the surface area not more than $1mm^2$. The sections of the leaves were soaked in 0.1% Mercuric Chloride. The sections of the leaf were then washed with sterile water and inoculated into 5ml of Nutrient agar medium slants which were incubated at 37^oC for 24-48 hrs.

B) Biochemical tests

Methyl Red test (2.Same for Voges Proskauer test): A loopful of the potential pathogen was inoculated in MR-VP broth for 24 to 48 hrs at 37^oC.

For MR test-4-5 drops of the methyl red indicator was added to the aliquot of culture.

For VP test: To the broth culture 10 drops of Baritt's reagent A was added and shaken followed by immediate addition of 10 drops of Baritt's reagent B .

3. Citrate test: To determine whether the bacteria could utilize citrate, a needle with inoculum was stabbed on a slant containing Simmon's citrate medium and incubated for 24-48 hrs at 37^oC.

4. Catalase test: To determine the ability of the bacteria to degrade hydrogen peroxide due to production of the enzyme catalase , the culture was streaked in nutrient agar medium and incubated for 24-48 hrs at 37^oC. 3-4 drops of the 3% hydrogen peroxide was allowed to flow over the entire surface of each slant culture.

5. Oxidase test: The ability to produce cytochrome oxidase was determined by addition of the test reagent p-aminodimethyl aniline oxalate,to colonies grown on a plate medium.

C) Koch's postulate

Koch's postulate demonstrates that a causative microorganism must be present in each case of a diseased animal or plant, and the isolation and re-inoculation of this suspected causative microorganism into the healthy host must result in the same disease again. Bacterial suspension was prepared by taking 5-6 colonies from the pure culture plate in 5ml of sterile water. The inoculum density of the bacterial suspension was determined by hemocytometer, which was $1.6x10^7$ cells/ml.

The two leaves for *Basella alba* were taken and divided into 4 quadrants. One was for control and the other was treated with the bacterial suspension. Four drops (each of 25 µl) of sterile water were given in each quadrant of leaf-1 that served as control and kept in a moist chamber; likewise four drops (each of 25 µl) of the bacterial suspension were given in each quadrant of leaf-2 as test and kept in a moist chamber in same manner.

The control and test plates for both the leaves were kept under the room temperature for 24 hours for observation.

D) Study of interaction between the potential bacterial and fungal pathogens of the plant Basella alba

The following experiment emphasizes on the interaction between the potential bacterial pathogen and the potential fungal pathogen isolated from the same source (leaf sections of Basella Alba). The bacterial species was characterized to be Gram positive short rod, while the fungal spores were consistent with that of *Bipolaris sp*. They were tested in presence of variable nutrient source. For instance, fungi are known to grow better in presence of sugar and bacteria are generally observed to grow better in presence of peptone. Thus, the effects of such nutrients on the growth of the pathogens were studied.

Spore suspension of the potential pathogen of *Basella alba* was prepared from slants containing fungal spores by washing the surface with sterile water and then sieving out the resulting solution. A bacterial solution was prepared in the same way. The spore suspension and the bacterial suspension were used to obtain the spore density and bacterial density respectively by using a Hemocytometer. 6% Dextrose sugar solution was prepared in 10ml of sterile water. 1.5% Peptone stock was also prepared in 10ml sterile water. Six grease free slides were labelled as C (Control), PC (Positive Control), NC (Negative Control) and TP 1, TP 2 and TP 3 (Test Plates) and the protocol cited in **Table 1** was followed during addition.

The suspensions were covered with cover slips and placed inside a moist chamber for 24hours at 31^{o}C.

Following 24hours incubation, slides were observed under microscope and the length of germ tubes and mycelia were measured and the number of germinating spores was counted.

E) Antibiotic sensitivity test

The antibiotic sensitivity test was carried on for the bacterial sample to check and compare the resistancy and sensitivity towards different antibiotics. This assay was done by disc diffusion test following Kirby-Bauer method with Ampicillin, Nitrofurantoin, Ceftazidime, Linezolid, Ciprofloxacin, Streptomycin, Clindamycin, Augmentin and Tetracyclin.

F) Identification of the bacterial sample 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) [5].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) [6]. 16S rDNA was amplified and sequenced using oligonucleotide primers complementary to highly conserved regions of bacterial rRNA gene.

DNA was isolated from the culture. 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.

Consensus sequence of **1369bp** 16S rDNA gene was generated from forward and reverse sequence data using aligner software (**Figure 8**).

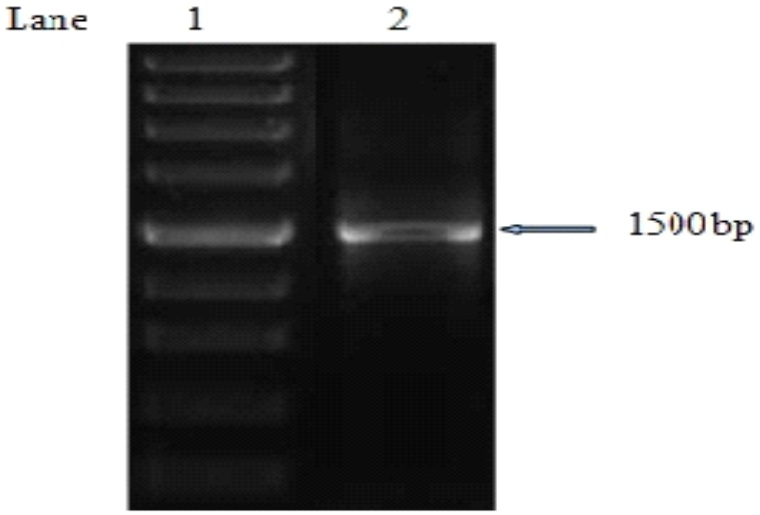
The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database.

Table 1: Protocol for addition of reagents during Slide bioassay

System	Spore suspension	Sterile H_2O	Bacterial Suspension	Peptone stock	Sugar stock	Total Volume	Peptone concentration (%)	Sugar Concentration (%)
C	5 µl	10 µl	0	0	0	15 µl	0	0
PC	5 µl	5 µl	0	0	5 µl	15 µl	0	2
NC	5 µl	5 µl	0	5 µl	0	15 µl	0.5	0
TP 1	5 µl	5 µl	5 µl	0	0	15 µl	0	0
TP 2	5 µl	0	5 µl	5 µl	0	15 µl	0.5	0
TP 3	5 µl	0	5 µl	0	5 µl	15 µl	0	2

Index: C (Control) = Spore suspension+ Sterile water,
PC (Positive Control) = Spore suspension+ Sterile water + 2% Dextrose,
NC (Negative Control) = Spore suspension+ Sterile water + 0.5% Peptone Stock,
TP 1 (Test Plate 1) = Spore suspension+ Sterile water + Bacterial Suspension,
TP 2 (Test Plate 2) = Spore suspension + Bacterial Suspension **+** 0.5% Peptone Stock,

TP 3 (Test Plate 3) = Spore suspension + Bacterial Suspension + 2% Dextrose,
TP 4(Test Plate 4) =Spore suspension (5µl) + Sterile water (5µl) +Fungicide (5µl)



[Gel Image 1]

Lane 1: DNA marker
Lane 2: 16S rDNA amplicon band
FIGURE 1: Gel Image of 16S r DNA Amplicon (bacterial sample)

RESULTS AND DISCUSSIONS

The leaf spot disease of Basella is a relatively old problem in the Global context caused by a large number of organisms. Leaf spot of Basella sp. is caused by *Cercospora spp*., which is one among the varied plant diseases reported in different part of the tropical world and also in the temperate zone of the Northern Hemisphere including the countries in North America including Hawaii [7]. In India the leaf blight of *Basella alba* has been reported at Umiam, Meghalaya, due to severe infection caused by *Rhizoctonia solani* resulting in appearance of characteristic lesions and gradual defoliation of whole plant[8]. Leaf Spot on *Basella alba* caused by a *Bipolaris sp*. in Florida was also reported .The necrotic lesions (up to 2 mm in diameter) round, semicircular, or irregular-shaped with grayish centers were surrounded by dark brown borders[9] . *P. Aphanidermatum* , a typical plant parasite of warm regions capable of causing root rot, soft rot, fruit rot or cottony blight of *Basella alba* has been isolated in Indonesia and Malaysia from *Basella sp*[10] especially when environmental conditions favor disease prevalence[11]. A severe brown leaf spot disease caused by *Curvularia lunata* [12] and *Verticillium* wilt caused by *Verticillium dahliae* [13]was also observed on the plants of Indian spinach or Malabar nightshade (*Basella rubra*).

In this present investigation, after 48 hrs, confluent creamy white mycelial growth was observed on and around region of the grey spot. It was thus hypothesized that a potential pathogenic organism has been successfully isolated from the infected leaf section but the degree of pathogenicity of the isolated pathogen is a topic of further investigation. Staining of pure colonies of sample B and sample F (**Table 2**) from diseased leaves showed that the bacteria was Gram positive , while the fungus to be *Bipolaris sp*(characterized by spore study (**Figure 2,3,4**). The results of the biochemical tests (**Table 3**) were found to be similar with the standard

biochemical tests of *Bacillus cereus* [14], where the culture showed positive results for Catalase and Voges-Proskaeur test and negative for Oxidase and Methyl red tests. Re-inoculation of isolated pathogens from diseased leaves to healthy leaves showed the growth of these pathogens with their disease symptoms as seen in diseased leaves of *Basella alba* (**Table 4**). The infection indicates that both the organisms are pathogenic to the *Basella alba* leaf. The re-inoculation of the fungal spore suspension led to infection again (68.75%) on the respective host leaves validating the Koch's postulates (**Figure 5(a) and (b)**.The bacterial sp. was also found to be pathogenic causing 35% infection in the leaf. The observation of slide-bioassay (**Table 5, Figure 6**) showed the presence of sugar increases the germination of conidia /spores and also the increased germtube length of fungi. From the observations recorded during the experiment the following deductions can be hypothesized:

The presence of bacteria reduces fungal growth which means they have an antagonistic interaction.

In presence of peptone the bacterial growth is enhanced and so when peptone is present along with bacteria the fungal growth is reduced in a greater number.

A dynamic interaction was observed in plate when fungi were present with bacteria in presence of sugar. Sugar is a source of nutrition for the fungus. So, in this plate though bacteria is present but the fungus utilized sugar as nutrition and growth has been observed here.

It further showed that fungicide (niacin) reduce germination as well as germtube length. Thus, the in-vitro slide bioassay showed that the use of fungicide though effective doesn't completely suppress the growth of fungal pathogens & is hazardous to health as well. Another important part of the study was to determine the sensitivity of the pathogenic Gram positive bacteria (Sample B) towards several antibiotics because bacterial diseases can be easily cured by applying antibiotics towards which the bacteria is sensitive. B. cereus produces betalactamases, and so is resistant to betalactam antibiotics; though susceptibility to clindamycin was reported earlier [15.].The result of disc diffusion test (**Table 6**) showed that the Sample B was resistant to those antibiotics for whom the zone of inhibition diameter was less than 20mm and sensitive to antibiotics for whom the diameter (zone of inhibition) was more than 20mm.Sample B is most sensitive to Clindamycin and least to Ampicillin (**Figure 7**).16S r DNA characterization showed the sample B to be *Bacillus cereus* strain IARI-B-24(**TABLE 7**).In the distribution of 109 blast hits on the query sequence of 1369 bp matched the alignment scores ≥200 (**Figure 9**) .Sequence producing significant alignments by BLAST closely matched to *Bacillus cereus* & different strains of Bacillus sp. were also found to be close to this species. Expected value of all the strains is 0.0 which depicts that all the strains are homolog to *Bacillus cereus* (**Table 7**),[16],[17],[18],[19].

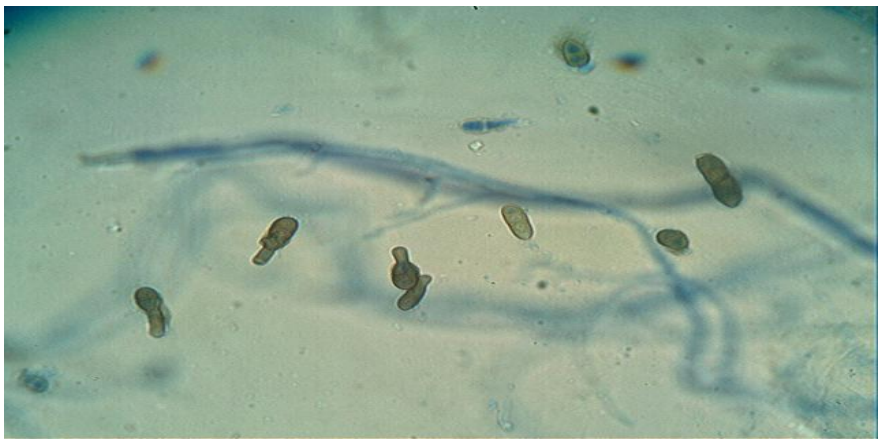


FIGURE 2: Spores of *Bipolaris sp.*

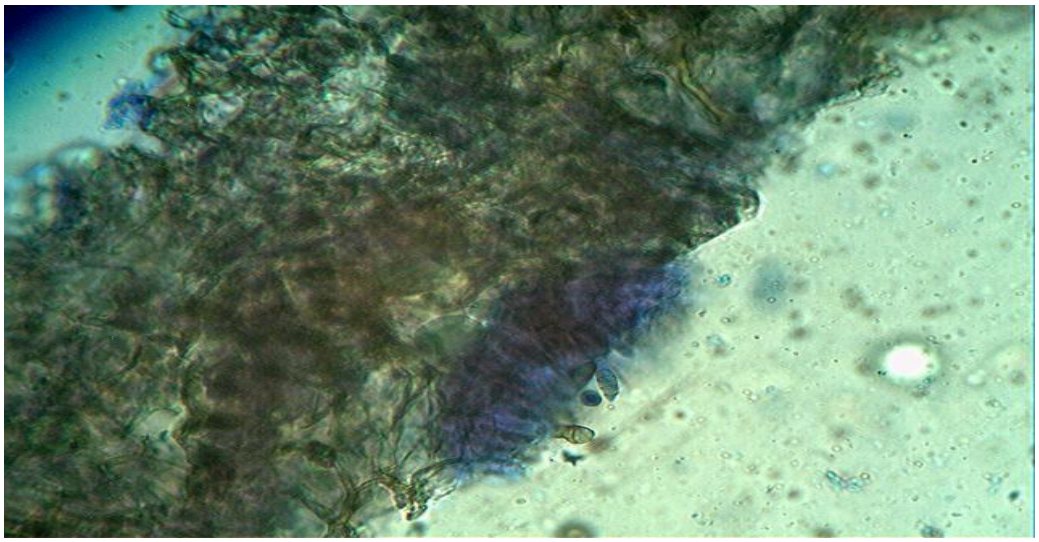


FIGURE 3: An infected area of the leaf showing fungal growth and presence of spores

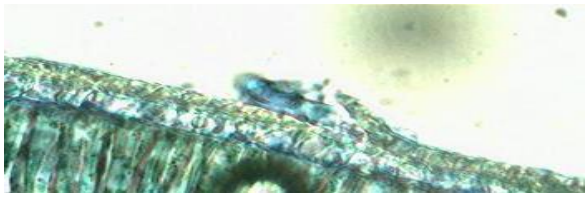


FIGURE 4: An *Appresoria* observed at the periphery of infected leaf. The different stages of fungal infection namely *appressoria*, ramifying hyphae, surface lesion along with conidia indicate the pathogenic nature of the fungi. Bulbous appressoria were seen attached to the cuticle from which infection tubes emerged and penetrated the leaf tissue.

NOTE: Fungal tissue stained with lactophenol cotton blue stain. Blue tissues are indicative of fungal mycelia.

FIGURE 5(a)

FIGURE 5(b)

FIGURE 5 (a) and (b): Fresh leaf exhibiting spots after re-inoculation with bacterial spore suspension (sample B)

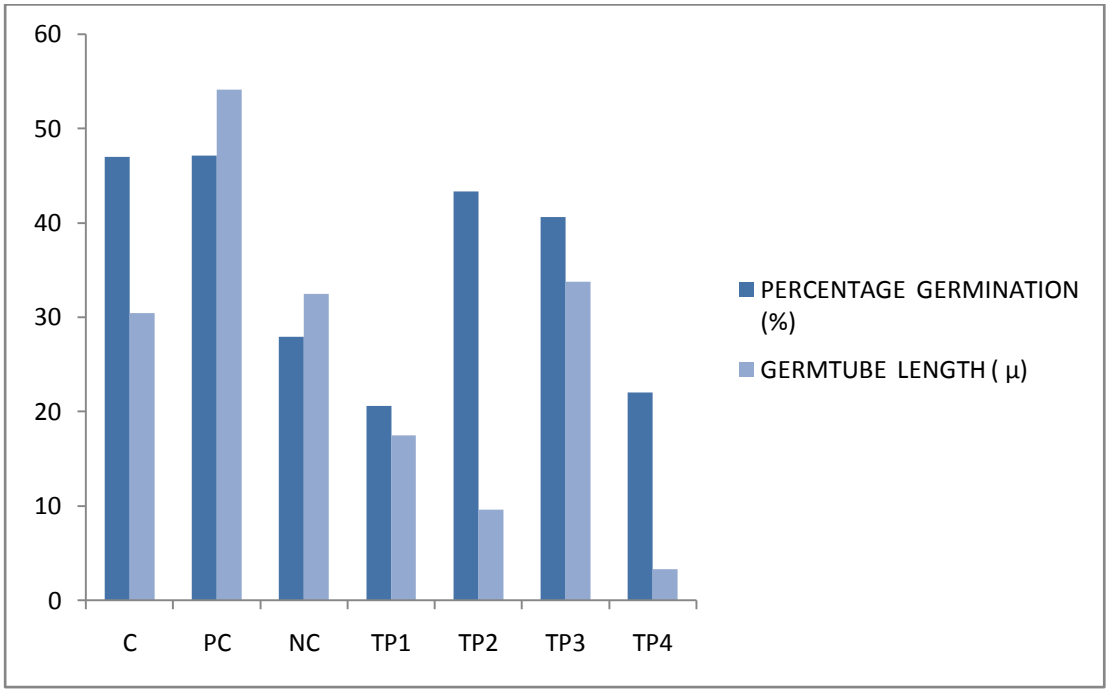


FIGURE 6: Graphical representation of slide bio-assay

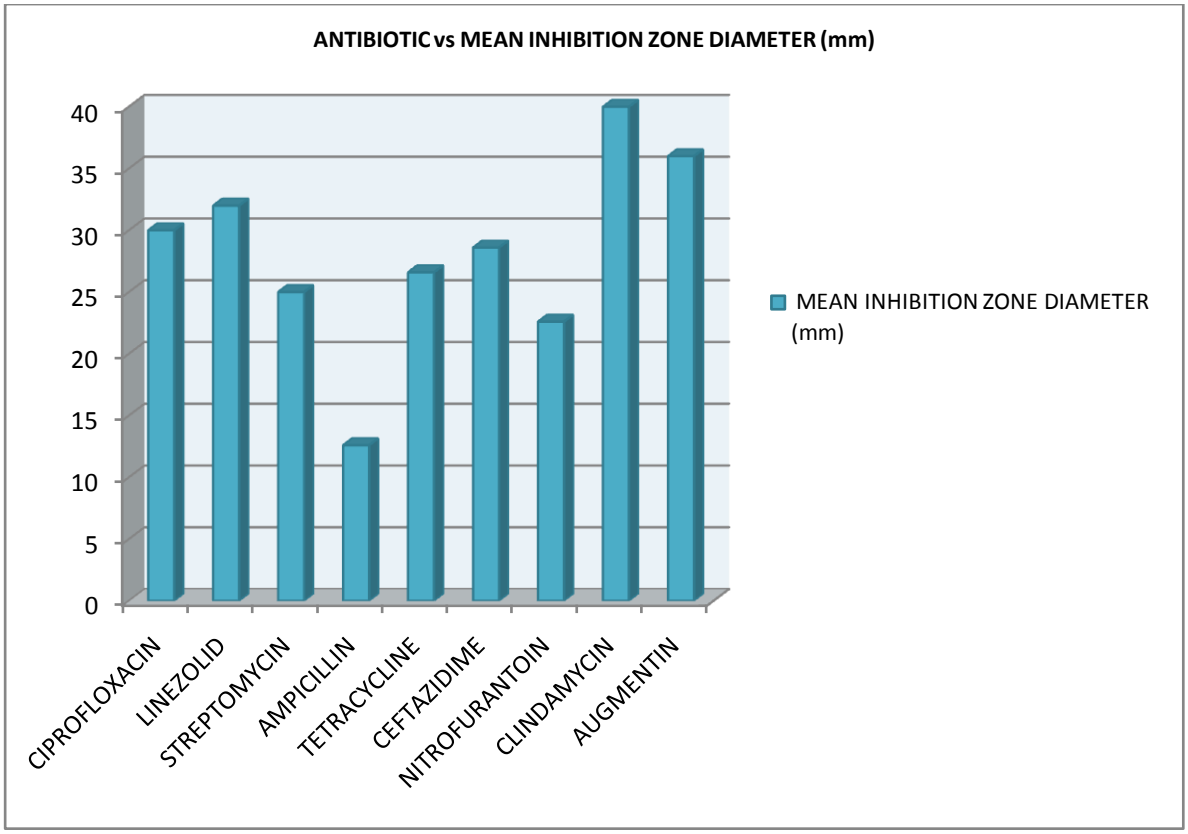


FIGURE 7: Graphical representation of antibiotic sensitivity.

Consensus Sequence S-1 (1369 bp)

AAGCTTGCTTCTATGACGTTAACGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACT
TCGGGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCA
CTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACC
TGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAG
GGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTA
AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAA
GCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCT
GTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCTGGTAGTCCACGCCGTAAACGATG
ACTGCTAACTCTTACACCGTTTCCCCCCTTTACTCCTCCACCTAACCCATTAACCACTCCCCCTCCCCACTACCC
TCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGAGCGTTCCCCTTCGGGGGACAGAGTG
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGA
TCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGA
GGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGC
TAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG
TTTGTAACACCCGAAGTCG

FIGURE 8: Consensus sequence of Sample B (1369 bp)

BLAST DATA: (Alignment view using combination of NCBI GenBank)

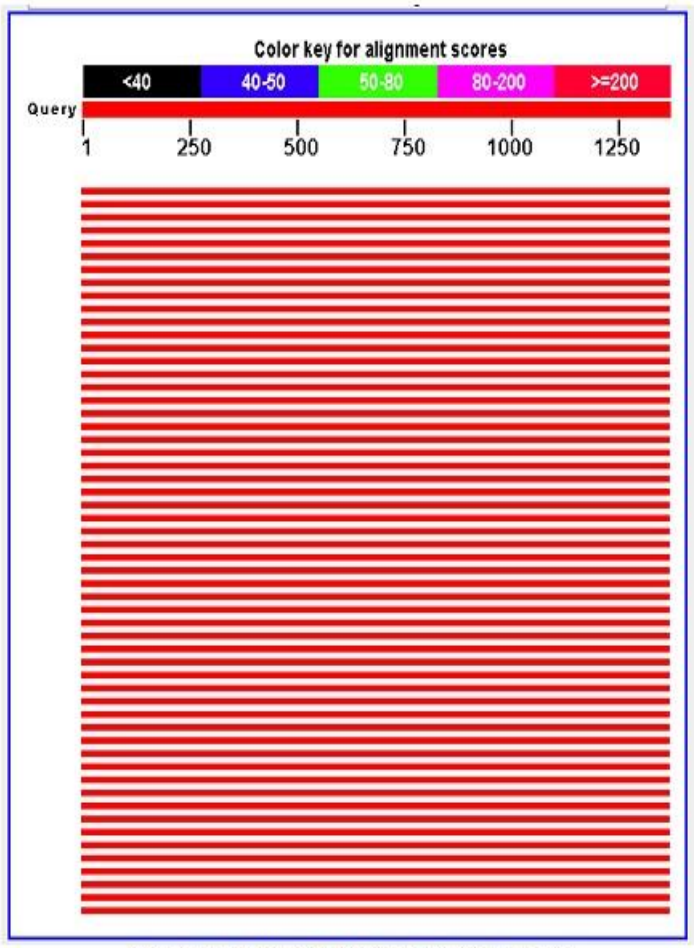


FIGURE 9: BLAST DATA: Distribution of 109 Blast hits on the query sequence

Table 2: Result of isolation and staining

Part of infection	Microscopic features	Sample	Microorganism(probable)
Leaf	Gram positive rods	B	*Bacillus sp.*
	Hypha are separated, brown in color, conidiophores are 4.5-6 µm wide, simple or branched, ascospores are of flagelliform in nature.	F	*Bipolaris sp.*

Table 3: Result of Biochemical tests

TEST	OBSERVATION
Oxidase test	Negative
Catalase test	Positive
Methyl red test	Negative
Voges- Proskauer test	Positive
Citrate utilization test	The culture turned blue in patches.(Positive)

Table 4: Results of Koch's postulates

a)Using sample B(Bacterial suspension)

Leaf species	Spots developed on control plates	Spots developed on test plates	Total drops of bacterial suspension added	Occurrence of the spots
Basella alba	0	5	20	35%

b) Using sample F (Fungal spore suspension)

Leaf Species	Spots developed on control plate	Spots developed on test plates	Total drops of fungal spore suspension added	Occurrence of the Spots
Basella alba	0	11	16	68.75%

Table 5 : Result of slide bio-assay. Study of interactions between the potential bacterial and fungal pathogens of the plant *Basella alba*

Name of Slides	Suspension of the microorganism	Solution	Percentage Germination	Length of germtube (µm)
CONTROL	Sample F suspension	Sterile water	47	30.44
POSITIVE CONTROL	Sample F suspension	Sterile water + 2% Dextrose	47.13	54.1
NEGATIVE CONTROL	Sample F suspension	Sterile water + 0.5% peptone stock	27.93	32.45
TEST PLATE 1 (TP1)	Sample F suspension + Sample B suspension	Sterile water	20.62	17.46
TEST PLATE 2 (TP2)	Sample F suspension + Sample B suspension	0.5% peptone stock	43.33	9.66
TEST PLATE 3 (TP3)	Sample F suspension + Sample B suspension	2% Dextrose	40.63	33.79
TEST PLATE 4(TP4)	Sample F suspension	Sterile water+ Fungicide(TAGO/ Niacin)	22	3.33

Table 6: Result of antibiotic sensitivity

Name of antibiotic	Mean zone of inhibition diameter(mm)
Ciprofloxacin	30
Linezolid	32
Streptomycin	25
Ampicillin	12.6
Tetracyclin	26.6
Ceftazidime	28.6
Nitrofurantoin	22.6
Clindamycin	40
Augmentin	36

Table 7: Sequence producing significant alignments (Source: The 16S rDNA gene sequence was used to carry out BLAST with the nr database of NCBI gen bank database.)

Sequence Producing Significant Alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN411389.1	Bacillus cereus strain IARI-B-24	2523	2523	100%	0.0	99%
JF837444.1	Bacillus sp. RJA3846	2523	2523	100%	0.0	99%
JX393073.1	Bacillus megaterium strain TDB-2	2518	2518	100%	0.0	99%
JX312585.1	Bacillus megaterium strain IARI-BC-13	2518	2518	100%	0.0	99%
JX312581.1	Bacillus megaterium strain IARI-BC-9	2518	2518	100%	0.0	99%
JX312579.1	Bacillus aryabhattai strain IARI-BC-6	2518	2518	100%	0.0	99%
JX293332.1	Bacillus megaterium strain S63	2518	2518	100%	0.0	99%
JX195647.1	Bacillus sp. enrichment culture clone JK7	2518	2518	100%	0.0	99%
JX195643.1	Bacillus sp. enrichment culture clone JK3	2518	2518	100%	0.0	99%
JX290312.1	Bacillus sp. enrichment culture clone AG.T.68.H4	2518	2518	100%	0.0	99%

CONCLUSION

There is no previous record of *Bacillus cereus* as a pathogen of *Basella alba* leaf. To our knowledge, this is the first report of a *Bacillus spp*. affecting Malabar spinach in India and at the same time showing antagonism with *Bipolaris spp*. From the study it was found out when there is combined infection of *Bipolaris spp*. along with *Bacillus cereus*(as identified by 16S rDNA analysis), the infection caused by *Bipolaris spp*. was of less intensity. Bacteria exhibiting antagonistic behavior or preparations can act as potential biological suppressers of phytopathogens. Combined infection of *Bipolaris spp*. and *Bacillus cereus* at the same time can serve as a potential biological control measure to prevent crop damage resulting in poor yield of *Basella alba*.

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