Original Research Article

Fungal Disease Detection of Post-Harvest Banana and its Eco-Friendly Quality Improvement Approach

ABSTRACT

Aims: The present study was designed to detect and characterize thecrown rot disease of post-harvestbanana (*Musa paradisiaca)* andalso develop an alternative quality improvement approach to improve banana shelf-life during storage period.

Study design: This study was an experimental laboratory design.

Place and Duration of Study: Disease infected bananas were collected from Rajshahi city, Rajshahi,Bangladesh in 2017 and the experiment had been conducted from April 2017 to April 2018.

Methodology: Different morphological, biochemical and molecular techniques (through 18s rRNA primer ITS4 and ITS5) were used to characterize and detect the liable fungi. Responsible fungi were subjected to antifungal activity screening test and*in vitro* antagonism test. Effect of carbendazim and kanamycin against the mycelial growth of the isolates was determined by disc diffusion method.Qualityparameters including disease incidence and severity, pH, TSS, TTA and AA of the treated banana were also analyzed after application of treatments in the packing stage through standard estimation techniques.

Results: Two fungi, isolated from the infected portion were further identified as *C.musae*and *L.theobromae*. *D. metel*and *A. sativum* extract was better in inhibiting mycelial growth of all the test pathogen in culture. *B. cereus* and *T. harzianum* moved and attached to fungal isolates, affecting mycelial growth and *A. sativum* extractsignificantly affecting conidial germination on artificial medium. Satisfactory mycelia inhibitory effect was recorded from kanamycin. Quality analysis after storage of banana showed minor measurable differences among treatments.

Conclusion: Post-harvest application of *A. sativum* extract (Conc. 25% w/v) improve the overall quality of harvested banana fruits and reduced the incidence and disease severity of crown rot to a level significantly lower than in fungicide treated or control fruits.

Keywords:*Musa paradisiaca, molecular technique,antagonism, mycelial growth, antifungal activity, quality analysis,disease severity.*

1. INTRODUCTION

Banana is one of the most important tropical crops and is affected by several fungal diseases, such as crown rot postharvest disease [1]. Ripe banana mixed with rice and milk is the traditional dish for Bangladeshi. Banana has several medicinal uses[2].Although banana fruits are highly demanded as nutritious and economically important fruits, they experience a different marketing problem[3].Crown rot is responsible for significant losses in banana fruits [1] and [4]. The fruit contains high levels of sugars and nutrients element, and their low pH values make them particularly desirable to fungal decayed[5].Crown rot begins with a mycelium development on the crown surface, followed by an internal

development[4]. Crown rot affects tissues of the crown, which unites the peduncle and subsequently development of fruit necrosis occur and main stalk decayed rapidly. Some common microorganisms were isolated from crown rot viz; *Colletotrichum musae, Lasiodiplodiatheobromae, Nigrosporasphaerica, Penicillium* spp., and *Aspergillus*spp[6]. Postharvest fungicidal treatments are applied to control crown rot disease, though severely affected banana fruits are still found in consumer markets [7]. There are different types of techniques for controlling the crown rot disease of banana and all are chemical control. There is no suitable report of antagonistic control system for crown rot disease of banana. Therefore, the study was designed to isolate the pathogen responsible for crown rot disease of storage banana along with its molecular detection and control of this devastating disease by antagonistic activities.

2. MATERIALS AND METHODS

2.1 Infected banana collection: Infected portion from the collected banana fruits were subjected to pathogen isolation [8].

2.2 Collection and extraction of plant material: Fifty grams of each milled plant specimens (*Datura metel*, *Faidherbiaalbida*, *Acacia catechu, Allium sativum*, *Solanum torvum*, *Solanum* spp., *Persicariastagnina*and *Azadirachtaindica*) were extracted by 250ml methanol solvent with continuous stirring for 15days using magnetic stirrer [9].

2.3 Collection and isolation of antagonistic agents*:* Pure culture of *Trichoderma harzianum* was obtained from the central laboratory of institute of biological sciences, University of Rajshahi, Rajshahi-6205, Bangladesh. *Bacillus* species was isolated using dilution method from rhizosphere soil samples with nutrient agar medium[10]. Gram staining test and a series of biochemical tests were performed for the characterization of the isolated bacteria [11].

2.4 Isolation of fruit rot fungi:Surface disinfected diseased parts were used to obtain pure culture of responsible fungi. The PDA plates were incubated at 25±1°C for seven days.

2.5Growth profiling of fungi: Potato Dextrose Agar (PDA), Czapek-Dox Agar (CDA), Sabouraud Dextrose Agar (SDA), Nutrient Agar (NA), Sabouraud Brain Heart Infusion Agar (SBHIA) and finally Corn Meal Agar (CMA) medium were used to examined cultural characteristics of the fungal isolates. Cotton blue staining slide was visualized for fungal spore detection[12].

2.6Molecular characterization

2.6.1 DNA extraction and PCR amplifications:After seven days of incubation mycelium from the two pure fungal isolates (Isolate-1 and Isolate-2) were separately subjected to isolation procedure. Here, Maxwell® 16 LEV Plant DNA Kit

(AS1420, Promega, USA) was used for the isolation of the genomic DNA. The isolated DNA was amplified through polymerase Chain Reaction (PCR) technique using universal primers ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') [13] and Hot Start Green Master Mix (Promega, USA). PCR was performed in a 50µl reaction mixture containing 25µl of Hot Start Green Master Mix (2X), 2.0 µL of each forward and reverse primer, 2.0 µL of genomic DNA and rest of the PCR water. The performing PCR program was as follows: pre heat at 95°C for 2 min, followed by 32 cycles of denaturation step at 95°C for 30 sec, primer annealing at 48°C for 30 seconds, primer extension at 72°C for 45 sec. After that, the temperature of final extension was at 72°C for 10 min and lastly, hold at 4°C for overnight. The amplicons were separated by 1% agarose (V3125, Promega, USA) gel electrophoresis.

Soil bacterium genomic DNA isolation was performed with cetyl-trimethyl ammonium bromide (CTAB) method [14]. PCR amplification of isolated soil bacteria was performed in the same technique of fungal DNA isolation and amplification using specific primers27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT -3') primer.

The quality and quantity of isolated DNA were checked by Nanodrop Spectrophotometer (ND2000, Thermo Scientific, USA). Finally, The PCR products were purified and used for sequencing analysis in Malaysia Ltd. via Invent Biotechnologies, Bangladesh. The sequenced data were analyzed using similarities of nucleotide sequences between isolates through the BLAST procedure (http://blast.ncbi.nlm).

2.7Pathogenicity test of isolated fungi: Wound inoculated and non-inoculated fruits (green banana, ladies' finger and apple fruits) were separately subjected to pathogenicity test [15].

2.8Effects of commercial fungicide: Two different conc. (25 and 50mg/disc) of fungicide (carbendazim) and standard kanamycin was tested against the fungal isolates for radial growth inhibition on PDA media using modified paper disc diffusion method under *in vitro* condition. The efficacy of a fungicide and kanamycin was expressed as per cent inhibition of mycelia growth over control.

2.9Antifungal activity screening: Antifungal activity screening was performed using moderate paper disc diffusion method [16]. About 50mg of the MeOH extract of each plant were weighted, dissolved in 1ml of the extraction solvent and then tested for antifungal activities.Kanamycin (50 mg $disc^{-1}$) was used as positive control.

2.10*In vitro* effect of *Allium sativum* extract against conidial suspension:Vogel's (minimal) medium[17] wasusedto detect the *in vitro* effects of garlic bulb extract against the conidial suspension of the isolates .10µl of garlic bulb extract and 90µl of the conidial suspension were mixed and the mixtures were added to the surface of depression slides or group slide. The slides were then incubated at 25°C for 24h. After that, the treated and control samples were spreading in petridish containing PDA medium and incubate overnight for evaluating antifungal activity.

2.11Determination of different quality parameters after *in vivo* application:

Artificially inoculated banana fruits were dipped into methanol extracts of *Allium sativum* (Conc. 25% w/v), while the control fruits were dipped into sterile distilled water [18]. Five fruits were used for each of the treatments. Standard estimation formulae were used to calculated percentage of disease incidence [19]. and disease severity [20].After 10 days of experiments fruit quality parameters including, pH,Total Soluble Solid (TSS), total titratable acidity (TTA) and ascorbic acid (AA) of the fruits were measured[21]. Ascorbic acid was determined using the dye method and expressed as mg $100g^{-1}$ of fresh fruits [22].

2.12Antagonistic assay: The antagonistic activity of *T. harzianum* wasevaluated against both the isolated crown rot fungi [23]. On the other hand, *B. cereus* at a conc. of 250µl/well was screened against test pathogen following agar well diffusion method.

2.13Statistical Analysis

All the above investigation of the present study was conducted in triplicate and repeated threes for consistency of results and statistical purpose. The data were expressed as mean±SE and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett 't' test using SPSS software of 10 version. $P<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Isolation of banana fruit rot fungi: Two types of fungi, were obtained from the infected portion, one forming pinkish white colony (Fig.1C) and the other showed gray to moss dark colony (Fig.1D) on PDA medium.

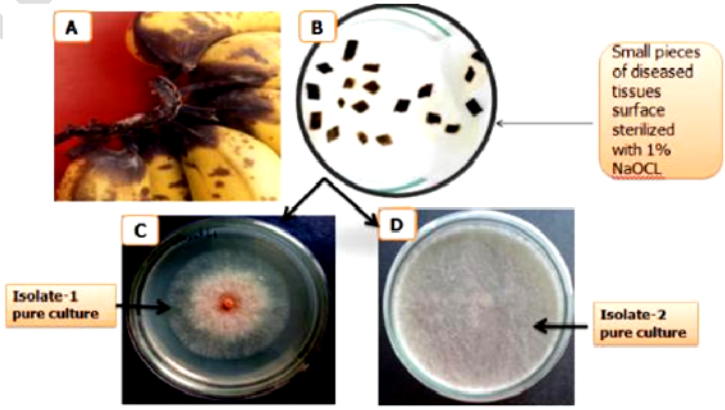

Figure-1: Collection of infected banana and isolation of responsible fungus

Legend: (A) Infected banana (B) Diseased tissue, (C) Isolate-1 and (D) Isolate-2.

3.1.2 Growth profiling of fruit rot fungi: Fungal isolates showed best aerial growth on PDA medium and cylindrical, septate and slightly rounded ends conidia were observed under light microscope (Fig.2: A-B) while no growth observed on CMA medium (Graph: C-F). Graph: C-F also represents colony diameter and dry mycelia weights of the fungal isolates. The optimum pH for mycelia growth of the isolates were pH 5.0-7.0 and 7.0 respectively (Graph: G).

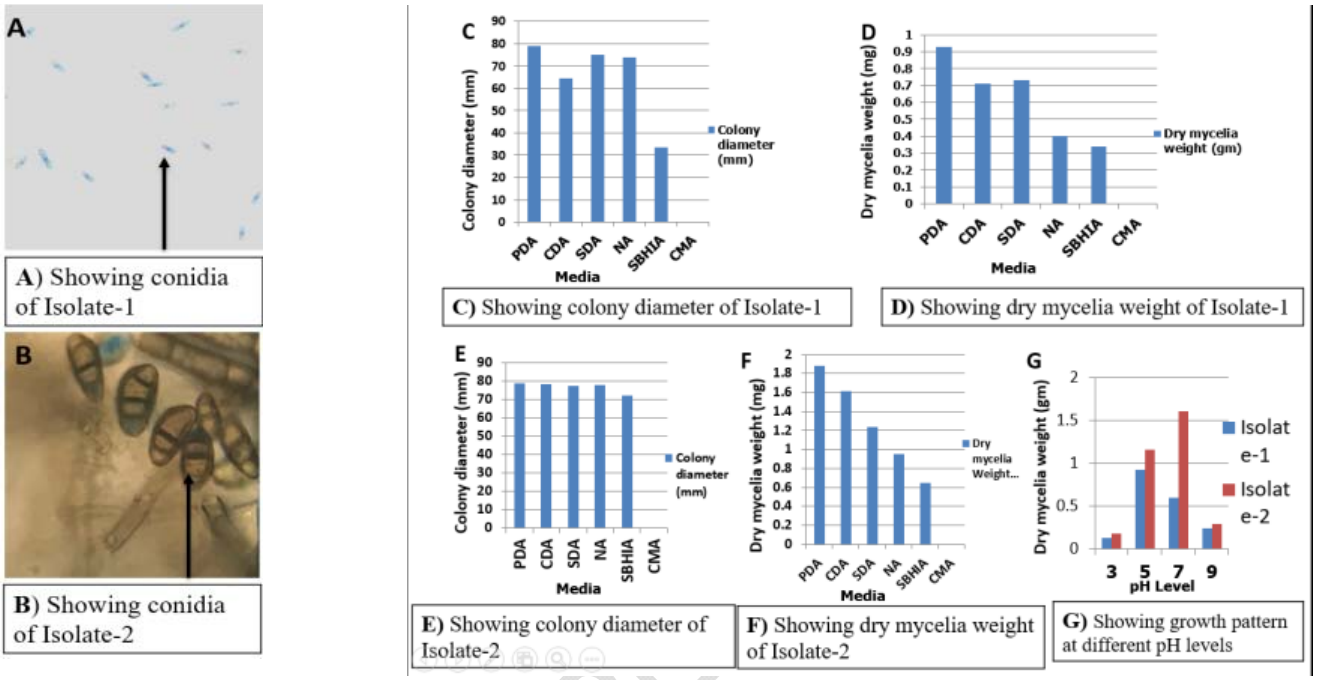

Figure-2: A & B showing Microscopic evaluation of isolate1 & 2

Graph C-G: Showing Growth profiling of fruit rot fungi.

Legend: Potato Dextrose Agar (PDA), Czapek-Dox Agar (CDA), Sabouraud Dextrose Agar (SDA), Nutrient Agar (NA), Sabouraud Brain Heart Infusion Agar (SBHIA), Corn Meal Agar (CMA)

3.1.3 Characterization of antagonistic agent:*T.harzianum* showed greenish colony morphology (Fig.3A) on PDA medium while isolated soil bacteria showed whitish creamy color and small to medium circular colony (Fig.3B) on LB agar plate.

Figure-3: Showing culture condition of *T. harzianum*and soil bacteria.

Legend:(A) *T. harzianum* (B) Soil bacteria (Whitish creamy color colony).

Morphological and biochemical test confirmed that,isolated bacterium was gram-positive, rod-shape and motile. Carbohydrate fermenting (TSI), Simmons citrate, KliglerIron Agar (KIA) test, and tween 80 hydrolysis tests positive, while it showed methyl red and mannitol test negative(Table 1).

Table 1. Morphological and biochemical characteristics of the isolated soil bacterium

Name of the test	Results
Gram staining	Gram-positive and rod-shaped
Motility	+(ve)
Simmons citrate	+ (ve)
Triple Sugar Iron (TSI)	+ (ve)
Methyl Red test (MR)	-(ve)
Klinger Iron Agar (KIA)	+(ve)
Tween 80 hydrolysis test	+(ve)
Mannitol test	-(ve)

Legend:+=positive (presence), - =negative (absence)

3.1.4Molecular characterization

3.1.4.1 PCR amplification: The genomic DNA isolated from the fungal isolates showed higher molecular weight and bright band on 1% agarose gel electrophoresis where 1kb DNA ladder was used as a marker. The universal primers, ITS-4 and ITS-5, were used to amplify a region of fungal genome named the 18S of ribosomal DNA gene of both isolate-1 and isolate-2. The PCR amplified fragments of both the isolates yielded two single band of around 600bp (Fig. 4). While 1492R and 27F primers were used to amplify a region of bacterial genome named the 16S ribosomal RNA gene. The bacterial isolates yielded a 1500bp high molecular weight single band on 1% agarose gel electrophoresis where 1kb DNA ladder was used as a marker (Fig.5).

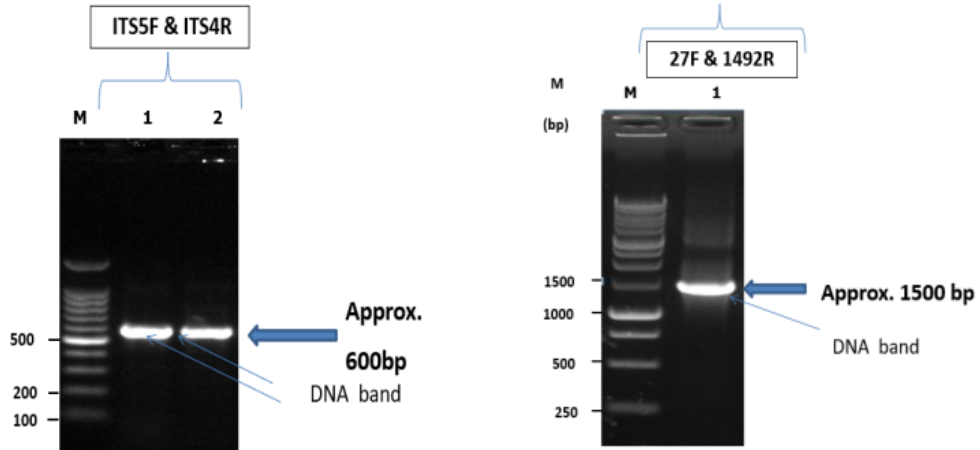

Fig.5: PCR amplification of soil bacteria using 1492R and 27F primers; (M) DNA ladder (Marker), **(1)** Isolated soil bacteria.

Fig.4: PCR amplification of both isolated fungi using ITS4/ ITS-5 primers; (M) DNA ladder (Marker), **(1)** Isolate 1 and **(2)** Isolate 2 fungi

3.1.4.1.2 Sequence analysis and BLAST: The data analysis revealed that the 18S of rDNA sequence of both fungal isolate (Isolate-1 and Isolate-2) showed 99% similarity with the original sequence of *Colletotrichum musae and Lasiodiplodiatheobromae*respectively. While the 16S of rDNA sequence of soil bacteria had 99% identity with *Bacillus cereus* isolate. The sequence data of isolate *C. musae* strain, *L*. *theobromae* strain and *B. cereus* isolate was deposited to the GenBank directly with access code of MH071339, MH084941 and MH119128 respectively (available to ENA in Europe and the DNA Data Bank of Japan).

3.1.5Pathogenicity test: Inoculated banana, ladies' finger and apple fruits showed typical crown rot symptoms, which were sunken, circular, necrotic, and dark-brown lesions indicated that fungal isolates were highly pathogenic. Later, whitish mycelia developed on the lesions (Figure 6).

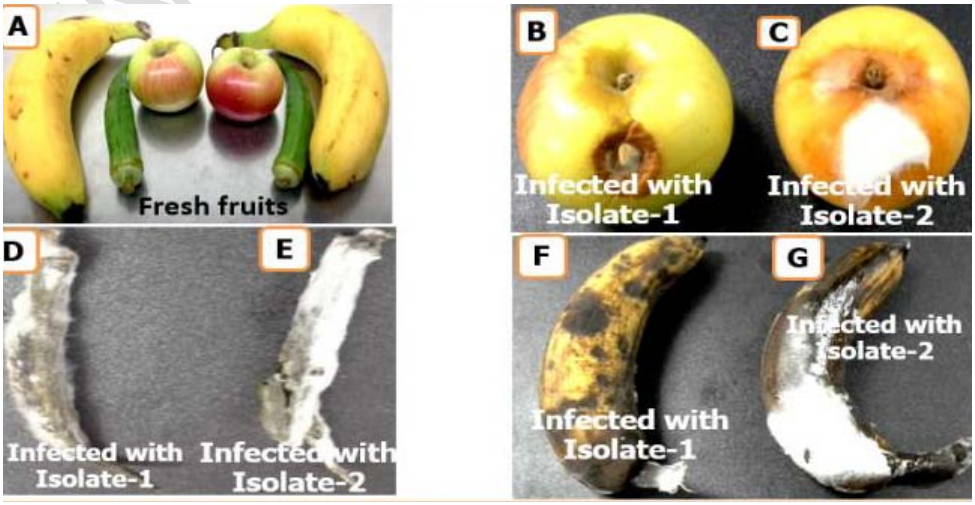

Figure-6: Showing pathogenic behavior of Isolate-1 and 2.

3.1.6Effects of commercial fungicide: Carbendazim inhibit highest 54% radial growth of mycelium compare to positive control against Isolate-1 while kanamycin inhibits 51% after 7 days of experiments which is very close to activity (Figure 7: A-D). Carbendazim change the normal color of the isolate-2 fungus and has little inhibition effect on the growth of the mycelium while kanamycin inhibits 11% (Figure 8: A-D).

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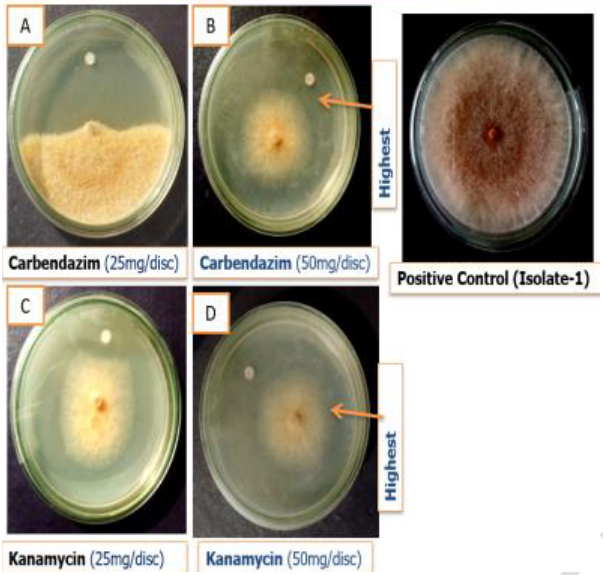


Figure-7: Effect of carbendazim and kanamycin against isolate-1

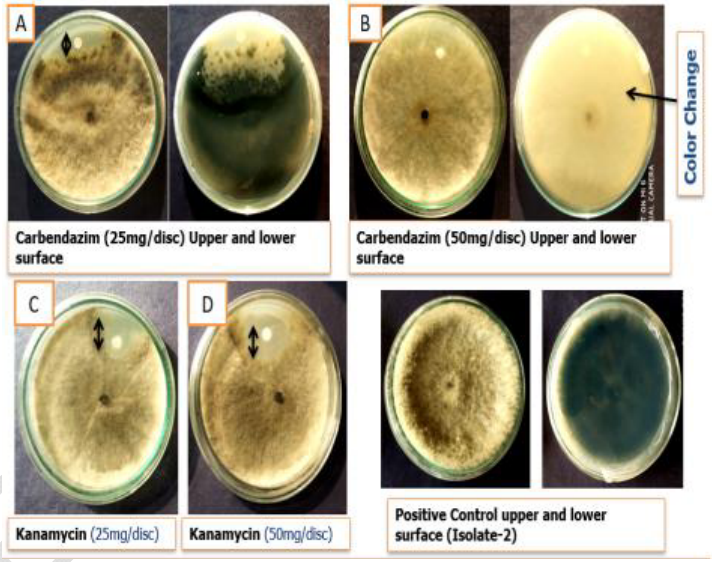
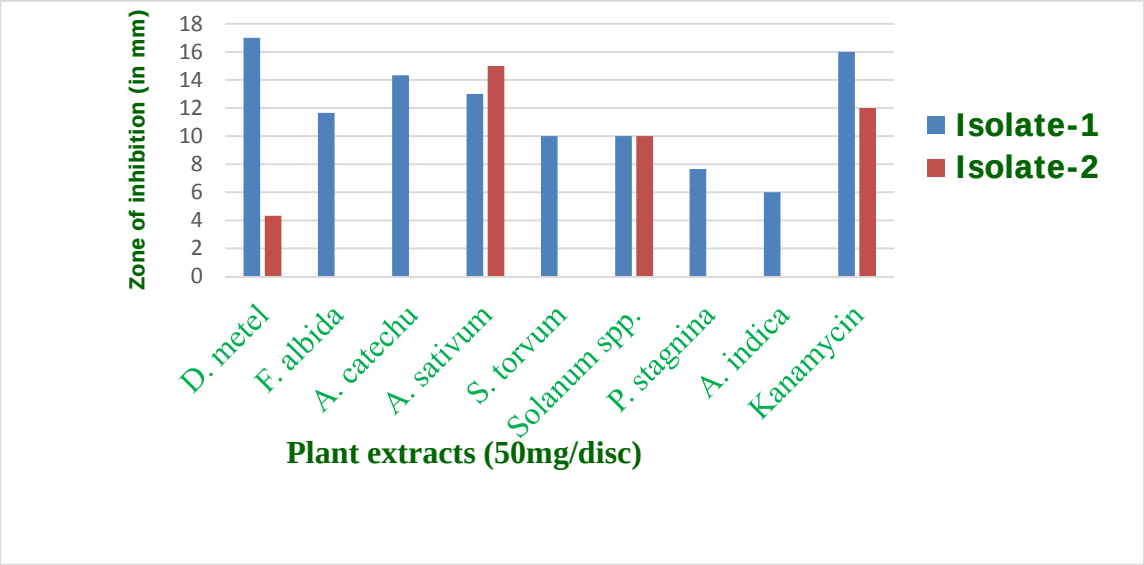


Figure-8: Effect of carbendazim and kanamycin against isolate-2

3.1.7Antifungal activity screening of plant extracts: In the experiment, methanolic extract of different plants showed inhibition at different levels against the aerial growth of two fungal isolates (Graph-H). The results revealed that the MeOH extracts of *D. metel, A. catechu, A. sativum* showed prominent inhibitory effects against Isolate-1 while only *Allium sativum* showed promising antifungal effect against Isolate-2 compare to standard kanamycin (positive control).



Graph-H: Showing antifungal activity of eight different plant extracts.

3.1.8*In vitro* effect of *Allium sativum* extract against conidial suspension: *Allium sativum* extract showed satisfactory antifungal activity against conidial suspension of both of the fungal isolates (Figure9).

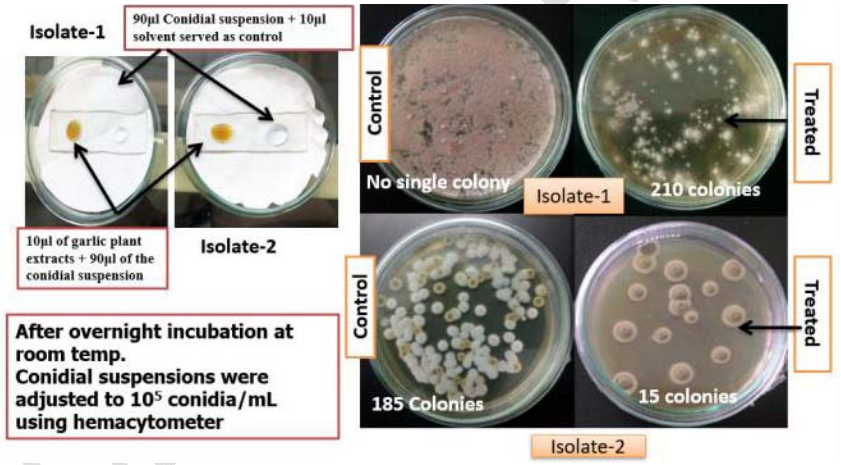


Figure-9: *In vitro* effect of garlic extract against conidial suspension of both the isolates.

3.1.9Determination of different quality parameters after *in vivo* application: The severity of fruit rot disease was equivalent to less than 1% fruit area affected in fruits treated with *Allium sativum* extract. Fruits in the untreated control ripened quickly and this led to the reduction of all the estimated overall quality of banana fruits (Figure10).

Figure-10: Results of *in vivo* evaluation of garlic extract and carbendazim.

Legend:(A-B) artificially inoculated with isolate-1, (C-D) artificially inoculated with isolate-2, (E-F) artificially inoculated with carbendazim (G-H) artificially inoculated with sterile distilled water.

However, 25% methanol extract of *A. sativum* resulted in TTA value comparable to that of fruits treated with carbendazim. Quality analysis after storage for total soluble solids, pH, total titratable acid and ascorbic acid for banana showed minor measurable differences among treatments (Table 2). This implies all the treatments, which increased fruit shelf-life and retain comparable color class of banana fruits, also preserved fruit internal quality. **Table-2: Quality parameters of banana after application of treatment:**

Treatments		**Disease severity (M±SE)**	**Diseases incidence (M±SE)**	**Results (Quality Parameters) (M±SE)**			
				p^H	**TSS**	**TTA**	**AA**
Allium sativum (25% w/v)	Isolate-1	11.18±0.90	49.66±1.24	5.30±0.41	18.83±0.62	0.43±0.40	5.03±0.73
	Isolate-2	25.09±1.38	79.9±1.593	5.03±0.02	17.23±0.40	0.35±0.44	4.40±0.41
Carbendazim		3.62±0.41	50.66±0.942	5.10±0.07	17.46±0.44	0.16±0.13	5.87±0.17
Control (SDW)		15.47±0.54	98.9±0.941	4.72±0.52	16.23±0.88	0.05±0.04	5.71±0.40

Legend: TSS= Total Soluble Solids (°Brix**)**; TTA= Total Titrable Acidity (%); AA= Ascorbic Acid (%); SDW= Sterile Distilled Water; ±= Plus/minus.

3.1.10Antagonistic activity: The most promising antagonistic activity was found when the isolates were co-cultivated with *T.harzianum*with at least seven days. On the other hand,soil bacteria (*Bacillus cereus*) showed some minor antagonistic activity against the tested fungi (Table3).

Table-3: Antagonistic activity against the isolated fungi

Antagonistic agent	Target fungus	Results (Zone of inhibition in mm) (M±SE)	
		4 days	7 days
T. harzianum	Isolate-1	69.66±1.24	84.33±0.47
	Isolate-2	45.33±1.24	70.66±0.81
B. cereus	Isolate-1	10.0±1.63	15.0±2.55
	Isolate-2	5.33±1.24	5.33±1.24

Legend:mm= Millimeter; M±SE= Mean Plus/minus Standard Error, Isolate-1: *C. musae***,** Isolate-2: *L. theobromae***.**

3.2 Discussion

Two types of fungi were obtained from infected tissues isolation technique, and later identified as *C. musae L. theobromae* according to the precise results of morphological and molecular approaches[24]. The optimum temperature was 25±1ºC and pH of isolate-1 and isolate-2 was 5.0 - 7.0 respectively[25]. Molecular analysis using ITS5F and ITS4R primer indicates approximately 99% similarity with the fungus *C. musa*e (isolate-1) and *L. theobromae*(isolate-2) responsible for post-harvest crown rot of banana [26]. Morphological test of isolated soil bacteria indicated that, it was gram positive and rod shaped. Molecular detection using 27F and 1492R primer and sequence (16S rRNA gene sequence) analysis of the isolated soil bacteria revealed, it was *Bacillus cereus* (99% similarity)[27]and [28]. Both the isolated fungus showed its high infection ability on fresh banana, apple and ladies' finger fruits[29]. Mycelia growth of Isolate-1 was significantly inhibited by methanol extracts (50mg/disc) of all the eight plant extracts while isolate-2 showed high sensitivity against *A. sativum*extracts compare to commercial fungicide and standard kanamycin[9]and [25]. From the result of commercial fungicide

and standard kanamycin test it can be concluded that kanamycin had a inhibition activity (inhibit 51% radial growth of isolate-1) which was more adjacent to the inhibition activity (inhibit 54% radial growth of isolate-1) of carbendazim. Complete control of crown rot pathogen is possible through application of benomyl, carbendazim and mancozeb[30]and [31]. Satisfactory *in vitro* antifungal activity of MeOH garlic clove extracts was observed against conidial suspension of both the isolates in the present investigation. *In vivo* evaluation of *A. sativum* treated fruit exhibit lowest disease severity and disease incidence compare to control. Fruit pH decreased in all treatments and storage temperatures during the storage period. A similar reduction in fruit pH in banana was reported previously[32].On the other hand, contrasting result for mango was reported[33]. Irregular changes of banana fruit pH during ripening were also reported[34].The values of the quality parameters of the *A. sativum* extract treated fruits showed some minor difference compare to fungicide treated banana fruits and sterile water treated banana fruits.The most promising antagonistic activity was found when the isolates were co-cultivated with *T.harzianum*with at least seven days. On the other hand,soil bacteria (*Bacillus cereus*) showed some minor antagonistic activity against the tested fungi. The effectiveness of *T. harzianum* and *Bacillus* spp. against mycelia growth of *L. theobromae* and *C. musae*was also reported[35], [36], [37] and[38]. The antifungal activity of *A. sativum* extract, and kanamycin 25% (w/v) was moderately comparable to antifungal activity of commercial fungicide which simply increase banana fruit shelf-life and maintain fruit quality. It also concluded that *D. metel* and *B. cereus* also showed some minor inhibition activity against Isolate-1.Establishment of biopesticides to prevent banana crown rot post-harvest disease, from the active antifungal component of effective plant extracts, antagonistic agents and kanamycin is one of the major future perspective of the present investigation.

4. Conclusions:

Advanced molecular technique-sequencing revealed the identity of fungal isolates as *C. musae*and *L. theobromae,* respectively which are the causal agents of crown rot diseases of banana in Bangladesh. The findings from the present study also suggest that *L. theobromae* was more prevalent than *C. musae*. This study suggests that *A. sativum, D. metel*extracts and kanamycin25%w/v (weight per volume) might be used as alternative quality improvement agent in the post-harvest stage. This study will help the researchers to uncover the critical areas of the inhibition mechanism of these bioagent that many researchers were not able to explore. Thus, a new theory on eco-friendly quality improvement approach of harvested banana may be arrived at.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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