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Characterization and Antagonistic Control Measures of *Pichia kudriavzevii* Yeast like Fungus in Brown Rot Disease of *Citrus sinensis* (L.) Osbeck

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Abstract: *Citrus sinensis* is one of the most important Vitamin C containing fruit all over the world which is badly infected by brown rot disease (BRD). The present research work was designed to isolate and characterize the pathogenic fungus responsible for BRD of *C. sinensis* as well as evaluation of its antagonistic control measures. The causal agent of BRD was isolated and characterized using physiological, morphological and molecular methods. The pathogenic fungus was isolated from the skin of sweet orange. The isolated fungus was grown in different nutrients media, temperature, pH, sugar concentrations and different carbohydrates. The highest growth of the fungi was observed in 5% fructose containing PD broth media at 37°C, pH 7.5 and 0.05gm/L NaCl concentration. PCR amplification of the isolated fungal DNA by internal transcribed spacer (ITS) region products showed approximately 650bp size of clear cut band. sequencing analysis showed that the amplified its region of the fungus (*GenBank Submission# SUB5672073*) has 99.80% similarities with the sequences of *Pichia kudriavzevii* isolate 11-577 its-1 partial sequence. In pathogenicity assay, artificially infected *P. kudriavzevii* fungus in lemon, malta and apple fruits showed a typical brown rot disease symptoms and the isolates showed similar size of 650bp clear band for PCR products. In antifungal activity assay, the methanol extracts of *Allium sativum* displayed highest 17.18±0.66 mm diameter zone of inhibition against the isolated fungus. *Rhizobium leguminosarum* and *Alternaria spp.* showed significant antagonistic effect in co-culture with the isolated fungus. The present research work would be helpful to control the devastating brown rot disease of *Citrus sinensis*.

Keywords: *Citrus sinensis*, BRD, ITS, *Pichia kudriavzevii*, Antagonism

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Introduction

Sweet orange (*Citrus sinensis* L.) is one of the fruit of the citrus species belonging to the family of Rutaceae, which reproduces asexually [1]. It is originated in southern China but now grown commercially worldwide in tropical, semi-tropical, and some warm temperate regions. Fruits qualities vary according to genetic background, environmental factors during growth and development, ripeness at harvest, postharvest conditions, and storage duration [2, 3]. As with other citrus fruits, orange pulp is the best source of vitamin C, providing 64% of the daily value in a 100g serving. Sweet oranges contain several types of phytochemicals, including carotenoids, flavonoids [4] and numerous volatile organic compounds producing orange aroma, including aldehydes, esters, terpenes, alcohols, and ketones [5].

Although sweet orange is highly demanded as nutritious and economically important, they experience a different marketing problem after harvesting and storage time [6]. Post-harvest decay can limit the extension of storage and shelf-life of fruits not only in Bangladesh but also all over the world. Major factors responsible for post-harvest loss of fresh fruits are mechanical damage, spoilage by fungi, bacteria, insects, other microorganisms and physiological deterioration [7]. One of the most limiting agents that influence the fruits economic value is its relatively short shelf-life caused by postharvest pathogens attack. The sweet orange fruit contains high levels of sugars and nutrient elements, and their low pH values make them specially targeted to fungal decayed [8]. Orange trees and fruits are susceptible to various pests and pathogens, including the Mediterranean fruit fly, numerous fungal leaf spots, blights, and root rots and viruses that can significantly reduce yields production [9].

Pichia kudriavzevii is very abundant in the environment and commonly found in soil, on the skins of fruits and even in fermented beverages. The fungus, *P. kudriavzevii* associated with a severe post-harvest brown rot disease of sweet orange in South Asian countries, like Bangladesh which can reduce the qualities of sweet orange as well as market values. To control brown rot disease of sweet orange, farmers are using enormous amounts of different synthetic and harmful fungicides which have a badly negative impact on human health and the environment. In Bangladesh, there is no suitable report for detection of the causal agent for post-harvest brown rot disease of sweet orange and its eco-friendly control system. Therefore, the present investigation was aimed to detect the causal organism of brown rot disease of sweet orange using morpho-physiological and molecular approaches as well as evaluation of effectiveness of some plant extracts and antagonistic agents like soil bacteria and fungi in controlling this devastating disease of sweet orange.

Materials and Methods

Source of plant materials

Post-harvest sweet oranges were collected from Shaheb Bazaar market, Rajshahi, Bangladesh which was subjected to pathogen isolation.

Collection of antagonistic agents

Two soil-borne bacteria (*Rhizobium phaseoli* and *Rhizobium leguminosarum*) and two non-pathogenic fungi (*Colletotrichum gloeosporioides* and *Alternaria spp.*) strains were collected from Microbiology Lab., Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh, which was previously isolated and identified. Collected bacteria and fungi were cultured on Luria-Bertani medium (LB) agar and potato dextrose agar (PDA) media respectively, storage at 4°C refrigerator (Walton, Bangladesh) for further experiments.

Isolation and culture of brown rot disease fungus

Sweet orange fruits surface were disinfected by washing them with 1% sodium hypochlorite for 1 min, and washed thoroughly with sterile distilled water. Disease infected parts were cut into small pieces and transferred to the surface of PDA (HiMedia, India) media containing plates using sterile forceps. From periphery of an actively growing culture on PDA medium, a mycelial disc of 6 mm diameter was cut and placed at center of sterile PDA medium containing petridish. The plates were incubated in dark room at 37°C for 7 days. All experiments were performed in triplicate for pure culture. The isolated fungus was sub-cultured on petridish containing PDA medium to grow and they were preserved in a refrigerator at 4°C for further experiments.

Morpho-physiological characterization of isolated fungus

Lacto phenol cotton blue staining

Lacto phenol cotton blue staining was performed as per the standard procedure. The fungal cultures were identified at genus level on basis of macroscopic characteristics like colony morphology, color, texture, shape and appearance and microscopic characteristics like conidia shape, hyphae color, septation, concentric zone, pigmentation, fruiting bodies or any other visible structures by cotton blue technique under a light microscope (LABOMED LX400, USA) at 40X magnification [10].

Effect of different media

Colony morphology, conidial morphology, spore characters and measurements were determined microscopically after incubation on PDA at 37±2°C for 7 days [11]. In the present study, actively growing isolates were placed at the center of the petridish, containing different semi solid media in each. The cultural characters of the isolates were studied on three different semi solid media, like Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) and Nutrient Agar (NA). The resulting growth of fungus mycelium was harvested and filtered through pre-weighed Whatman No.1 filter paper and washed thoroughly with distilled water. The isolated mycelium was dried at 40°C for two days in hot air oven and the dry weights were recorded. Colonies on each medium were compared for their overall colors, colors of conidia, reverse colors, form, margin, elevation, and dry weight.

Effect of different pH levels

The optimum pH for mycelial growth was determined on PD broth, where pH was adjusted as 3, 5, 7.5 and 9. After sterilization, the culture bottles were kept in laminar flow hood for cooling down. After cooling down, five days old isolate culture growing on PDA plates were separately punched aseptically with a 7 mm diameter sterile cork borer. The fungal discs were then put on the centre of all culture vessels containing PD broth. After seven days of inoculation, colony morphology and mycelial dry weight was recorded using electric balance.

Effect of different temperature

Isolated fungus was cultured at different temperatures like, 4-5°C, 21-25°C and 37°C to evaluate the best condition of the isolated fungus growth and development. Using PDA medium, the fungus was cultured and incubated at 4-5°C, 21-25°C and 37°C. After 7 days of incubation, mycelium was collected, dried and weight was measured using electric balance.

Effect of carbohydrates

To standardize the effects of carbohydrates on the growth of fungus, 5% different carbohydrates such as fructose, glucose, lactose and maltose were used. At first PDA medium were prepared in four culture vessels then mixed 5% glucose, lactose, maltose and fructose of each of the vessels. The isolate was inoculated in different carbohydrates treated culture vessels and were incubated in a dark room at 37±2°C for 7 days. After the fungus grown properly, the mycelium was isolated, dried and weighted in an electric balance for statistical analysis.

Effects of NaCl

To detect the effects of NaCl, PDA medium was prepared and the different amount of NaCl such as 0.01gm, 0.05gm, 1gm, 1.5gm and 2gm per dish was added. The isolated fungus was cultured in PDA medium containing different amount of NaCl and the culture vessels were incubated at 37±2°C for 7 days. After 7 days of incubation the dry weight of the fungus mycelium was taken using an electric balance for statistical analysis.

Molecular characterization

DNA extraction and purification

Total genomic DNA was isolated from isolated fungus fruit bodies using all Prep Fungal DNA/RNA/Protein Kit (QIAGEN, Japan) followed by their 'Quick start-protocol'. The DNA was suspended in TE buffer and quantified using a spectrophotometer then electrophoresed on 1% agarose gel by comparison with DNA samples of known concentration.

PCR amplification

The isolated fungal DNA was subjected to the PCR amplification using universal primers ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') [12]. PCR amplification was performed in a 25µl reaction mixture comprising 9.5 µl deionized

distilled water, 12.5 µl hot start green master mix, 1 µL of each forward and reverse primer (Promega, USA) and 1µL cDNA (con. 25-65 ng/µl). PCR amplification for ITS regions was performed in a Gene Atlas (Astec, Japan). PCR cycles started with an initial pre-heat at 95°C for 2 minutes, followed by 32 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C and a 45 seconds, extension at 72°C, with a final extension at 72°C for 5 min. Amplicons were separated by agarose gel electrophoresis (1%) in 0.5X Tris/borate/EDTA buffer. A 1kb DNA Ladder (Promega, USA) and DNA binding dye (Promega, USA) was used for the visualization and identification of PCR product. DNA was quantified electrophoretically and spectrophotometrically using NanoDrop Spectrophotometer (ND2000, Thermo Scientific, USA). PCR products were purified using the SV Gel and PCR Clean up System (Promega, USA.), according to the manufacturer protocol (PCR purification kits, Promega, USA).

Sequencing, alignment and BLAST

Previously isolated fungal DNA was used to performed PCR amplification using ITS4R and ITS5F primers. Products of PCR reactions were analyzed in Invent Biotech, Bangladesh. The 18S sequences were obtained from GenBank database at the National Centre for Biotechnology Information (NCBI) and aligned using the Pileup program available as part of the GCG package. The DNA sequences of the fungal isolates were compared with the sequences available by the Basic Local Alignment Search Tool (BLAST) in the NCBI, Gen-Bank database (<http://www.ncbi.nlm.nih.gov>). The sequences were aligned together with those of reference taxa retrieved from public databases. All the sequences were also aligned with ClustalW program for constructing a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining method. The numbers on the nodes correspond to the percentages, with which a cluster appears in a bootstrap test based on 54661 genes. The bars denote the relative branch length. The ITS region was identified from NCBI GenBank database.

Pathogenicity test

In vivo pathogenicity capability was evaluated by Ziedan *et al.* [13] methods. For the pathogenicity test, healthy green lemon, malta and apple fruits were surface-sterilized with 70% ethanol, and wounds were made in each fruit using sterilized wooden rod. In each fruit, each wound was inoculated with mycelial plugs (3mm) from a 7 days old culture of each isolate, and one was treated with uncultured pure PDA medium as a control. Inoculated fruits were covered with poly bags, and incubated at 37±2°C for 7 days [14]. Fungi were collected from infected samples, cultured, DNA isolation, purification and PCR were performed, respectively. Three replicates were made for each treatment.

Biological control assessment

Antifungal activity assay

The antifungal activity of the plant extracts were tested by disc diffusion method [15] against isolated fungus. Selected plant specimens namely, *Psidium guajava*, *Allium sativum*, *Coccinia grandis*, *Hibiscus rosa-sinensis*, *Moringa oleifera* and *Cassia alata* were collected, dried under

shade, milled and extracted with methanol using previously reported method [16]. Here, 20 ml PDA medium was plated in each petridish. When the plated medium was cooled, it was inoculated with isolated fungus. Filter paper discs (6 mm in diameter) impregnated with the concentration of 200µg/disc for each of plant extract was placed on test organism-seeded plates. Methanol was used to dissolve the extract and was completely evaporated before application on test organisms seeded plates. Blank disc impregnated with solvent methanol followed by during off was used as negative control. The activity was determined after 7 days of incubation at $37\pm 2^{\circ}\text{C}$. The antifungal activity of the plant extracts was evaluated and the results of diameter of zone of inhibitions were measured in millimeter (mm) scale.

Antagonistic assay

Antagonistic activity was determined by disc diffusion method [17]. The discs (6mm) were taken into the screw capped tube and sterilized in an autoclave at 121°C for 20 minutes to ensure sterilization. To evaluate the antagonistic efficiency of two isolated soil borne bacteria and two non-pathogenic fungi were co-culture with the isolated fungus. From previously cultured antagonistic agents (bacteria and fungi) were taken with help of micropipette or wire loop and applied on paper discs one by one and the culture dishes were incubated at 37°C for 7 days. After incubation, the antagonistic activity of the test samples was determined by measuring the diameter of growth of soil bacteria and non-pathogenic fungi against the isolated fungus with a transparent scale.

Statistical analysis

All the above investigations of the present study, data were subjected to analysis of variance (ANOVA) with for a completely randomized design with three replicates. The data were expressed as mean and standard error (Mean $\pm$ SE) using Microsoft Excel 2013 version. The means were separated using Duncan's multiple range tests at $P=0.05$, where the F-value was significant.

Results

Morpho-physiological characterization

Isolation of fungi

The fungi were isolated from the infected fruit of sweet orange. At the incubation period, the fungal colony was appeared due to mycelial growth of the fungi. Isolated fungus initially produced cottony white fluffy growth (Figure 1 A) on PDA medium, which covered the petridish within 5 to 7 days at 37°C . Under the lower surface of the dish, complete blackish background was observed due to pigmentation by the fungus in medium. Hyaline branched mycelium produced pycnidia with in 48hrs. which produced hyaline elliptical single celled pycnidiophores.

Microscopic identification of cotton blue staining

The development of mycelia on PDA medium was considerable important for the detection of colony morphology. In PDA medium, the fungus was white or blackish white in morphology.

After cotton blue staining the fungus was observed that, the conidia was very long and multi-septate, conidia were pale olivaceous brown and septate (Figure 1B) under electronic microscope.

Figure 1: Showing the isolated fungus and cotton blue staining (A) isolated fungus culture (white) at day 7 and (B) microscopic view of conidia

Growth effect in different media

Among the different media for isolated fungus culture, PDA medium showed the highest 2.59gm weight of mycelium per dish, while SDA medium showed the lowest 0.27gm of mycelium weight after day 7 of dry weight. NA medium showed moderate effect in the isolated fungus. Growth profiling effects of different media for isolated fungus are presented in Graph 1.

Graph1: Growth effect of mycelium in different media

Growth effect in different pH

The isolated fungal sample was grown at different pH ranges. At pH 3, 5, 7.5 and 9, the colony radial growth was observed with 0.02gm, 0.23gm, 0.39gm and 0.19gm dry weight after 7 days of culture, respectively having PD broth medium. The highest dry weight of mycelium was found to be 0.39gm at 7.5 pH followed by 0.23gm at 5 pH. On the other hands, the lowest dry weight of

mycelium was found to be 0.02gm at pH 3. Different pH levels effects on isolated fungus grown and their dry weight of mycelium are showed in Graph 2.

Graph 2: Growth effect of mycelium in different pH levels

Growth effect in different temperatures

Temperature is a vital factor which controls the enzymatic activities of fungus growth and development. The isolated fungal sample was grown at different temperature ranges to check the effect of temperature on their growth. At 10-13°C, 21-25°C, 37°C and 45°C, the colony radial growth was observed with 0.00gm, 0.19gm, 0.36gm, 0.48gm and 0.09gm dry weight, respectively (Graph 3). The highest dry weight of mycelium was found to be 0.48gm/dish at 37°C followed by 0.36gm/dish at 21-25°C at PD broth medium. On the other hands, there was no fungus growth and development was observed at 4-5°C. Growth profiling effects of different temperatures in isolated fungus are given in Graph 3.

Graph 3: Growth effect of mycelium in different temperatures

Growth effect in different carbohydrates

Different types of carbohydrates has effective role in growth and development of fungal mycelium. Different types of carbohydrates were treated at 5% level. The highest dry weight of mycelium was observed 1.41gm/dish in fructose containing PD broth medium followed by 1.306gm in glucose containing medium. On the left hand, the lowest growth of fungus was found to be 0.26gm /dish in maltose having PD broth medium. Growth profiling effects of different carbohydrates on the isolated fungus are presented in Graph 4.

Graph 4: Growth effect of mycelium in different carbohydrates

Effect of NaCl in mycelium growth

Different concentrations of NaCl played an important role in growth and development of fungus. The highest dry weight of mycelium was found to be 0.72gm per dish at 0.05gm NaCl containing PD broth medium at 37°C for 7 days of incubation followed by 0.49gm mycelium at 1.0gm NaCl/dish containing PDA medium at 37°C after 7 days of incubation. The lowest weight of mycelium was observed 0.33gm at 1.5gm concentration of NaCl per dish. Effect of NaCl concentrations on mycelium growth of fungus are showed in Graph 5.

Graph 5: Growth effect of mycelium in different weight of NaCl

Molecular characterization

PCR amplification

The primers ITS4 and ITS5 pair gave rise to amplification of fungal DNA products of different intensity. In gel electrophoresis, PCR products showed a clear cut band of 650bp approximately comparison of 1500bp DNA marker. Whereas, negative control of PCR products did not show any band under UV spectrophotometer. PCR amplification results are showed in Figure 2.

Figure 2: Photograph of ITS-DNA amplified band for isolated fungal pathogenic strain. M: 1 kb DNA ladder (Marker), I: isolated fungus and N: negative control

Sequencing, alignment and BLAST

Identification of the fungal isolates was based on molecular genetic analysis using the initials ITS. The universal primers ITS4 and ITS5 were used to confirm the identity of the fungal isolate and draw phylogenic relationship, the 'ITS' region of ribosomal RNA gene was amplified and sequenced (GenBank Submission # SUB5672073). The 650bp sequenced nucleotide sequence was performed for a BlastN search in GenBank (<https://www.ncbi.nlm.nih.gov>). It was revealed that, the strains had approximately 99.80% similarity with *P. kudriavzevii* (Figure 3A). The data was aligned and prepared a phylogenetic tree that showed the other similar species of *P. kudriavzevii*. Phylogenetic tree of ITS region of the rRNA gene with other various yeasts are presented in Figure 3B.

Figure 3: Sequencing data and phylogenetic tree showing the taxonomic positions of different fungal strains isolated from postharvest citrus fruits, based on the ITS sequences and other closely related species available from NCBI. (A) Blast result and (B) the phylogenetic tree.

Pathogenicity test

In pathogenicity test, isolated fungus was inoculated on the surface of lemon, malta and apple fruits, which showed typical brown rot disease symptoms of sunken, circular, necrotic, and white lesions after incubation at 37°C for 7 days. Later, darkish mycelia developed on the lesions with subsequent dark colored conidial structures. Morphological as well as cultural characters of isolated fungi (Figure 4 A-C) were compared with those of previously isolated fungus. Re-isolated fungi were cultured, DNA extracted and PCR was performed using ITS primers. The PCR products showed clear cut band of 650bp length size in case of previously isolated fungus (as positive control), lemon, malta and apple infected fungi. Whereas the negative control of PCR products did not showed any band. They also showed similar results for sequencing (Data not given). PCR results of pathogenicity effects are presented in Figure 5.

Figure 4: Brown rot disease incidence of sweet orange from room cultivation 20 days after infestation.

A: lemon, B: Malta and C: apple fruits

Figure 5: Photograph of ITS-DNA amplified band for fungal pathogenic strain. M: Marker, P: isolated fungus as positive control, A: lemon, B: malta C: apple infected fungus and N: negative control.

Biological control assay

Effect of plant extracts against the isolated fungus

In this study, the antifungal activity of six different plant extracts against the isolated fungus. In antifungal activity, *Allium sativum* showed the highest 17.18 ± 0.66 mm diameter of zone of inhibition followed by 15.33 ± 0.72 mm diameter of zone of inhibition zone showed by *Psidium guajava* at the concentration of $200 \mu\text{gm}/\text{disc}$ against the isolated fungus. On the other hands, the lowest inhibition zone was 10.33 ± 0.82 mm diameter, showed by *Coccini agrandis* at the same

concentration. Moreover, *Hibiscus rosa-sinensis*, *Moringa olerifera* and *Cassia alata* also showed significant antimicrobial against the isolated fungus. Antimicrobial effects of different plant extracts against the isolated fungus are presented in Graph 6 and in Figure 6.

Graph 6: Effect of different plant extracts against the isolated fungus

Figure 6: Effect of different plants extracts against the isolated fungus (A) *Psidium guajava* (B) *Allium sativum* (C) *Coccinia grandis* (D) *Hibiscus rosa-sinensis* (E) *Moringa oleifera* and (F) *Cassia alata*, NC=Negative control

Antagonistic test

Two different soil born bacteria (*Rhizobium phaseoli* and *Rhizobium leguminosarum*) and two different nonpathogenic fungi (*Colletotrichum gloeosporioides* and *Alternaria spp.*) were co-cultured against the isolated fungus. Both soil borne bacteria and nonpathogenic fungi showed

significant inhibition zone against the isolated fungus. Comparisons effects of different antagonistic agents against the isolated fungus are presented in **Figure 7**.

Figure 7: Antagonistic test of soil borne bacteria and fungi against the isolated fungus. (A) *Rhizobium phaseoli* (B) *Rhizobium leguminosarum* (C) *Colletotrichum gloeosporioides* (D) *Alternaria* spp.

Discussion

The present study was designed to isolate and characterize the causal agent of brown rot disease of *Citrus sinensis* and evaluate an eco-friendly controlling system of the isolate. The isolated fungal pathogen was identified by morphological, physiological and molecular characteristics. Isolated fungus was found to be blackish white and cottony to fluffy mycelia on PDA medium. In microscopic examination, conidiophores were observed as pale brown to blackish background due to pigmentation and the conidia were straight to slightly curved, thin walled and multi septate under light microscope. Choi *et al.* [18] showed similar morphological and microscopic results for a fungal isolate from nuruk and the pathogen was identified as *P. kudriavzevii*. Jahan *et al.* [19] observed cylindrical, septate and slightly rounded ends conidia under light microscopic evaluation in case of crown rot disease of banana which supports our present findings. In the growth profiling of the present fungal isolate, PDA medium was found to be best for growth and development. The highest mycelium (dry) growth rate of the isolated fungus was observed in PD broth medium having 1.41gm/L fructose and 0.05gm/L NaCl containing pH at 7.5 and the at the temperature of 37°C. The same result was reported by Choi *et al.* [18] in *P. kudriavzevii* growth for ethanol production from nuruk and Hasan *et al.* [20] for growth and development of *Cercospora* spp. which confirm our present findings. *P. kudriavzevii* showed strong thermotolerance [21]. On the contrast, Oberoi *et al.*, [22] and Greppi *et al.* [23] reported as high as 45°C and Choi *et al.* [19] at 44°C for best growth and development of *P. kudriavzevii*. Hasan *et al.* [20] reported the best response for growth and development of a fungal stain in PDA medium

having 1.41gm/L fructose which supports our present investigation. On the contrast, Choi *et al.* [18] reported the best carbon assimilation patterns of the isolated *P. kudriavzevii* MBY1325 in glucose and glycerol containing media.

For the confirmation of the present isolate as *P. kudriavzevii*, PCR and gene sequencing was performed using a pair of universal primer. The primers ITS4R and ITS5F pair gave rise to amplification of fungal DNA products of different intensity. In the present investigation, PCR products showed a clear cut band of 650bp in gel electrophoresis. The universal primers were used to confirm the identity of the fungal isolate by gene sequencing and draw phylogenic relationship. The 'ITS' region of ribosomal DNA was amplified and sequenced. A BlastN search in GenBank using the 508bp ITS sequence revealed that the strain showed 99.80% similarity with sequences of *P. kudriavzevii* isolate 11-577 ITS-1 of partial sequence. Chowdhury *et al.* [24] reported 100% similarity with ITS sequence of *Fusarium oxysporum* which was very similar to our present results. Phylogenetic tree that showed the other similar species of *P. kudriavzevii* fungus. For more confirmation, pathogenicity test was done using some fresh fruits. After two weeks later newly grown fungi was isolated. Isolated fungus from the diseased fruits was cultured as well as fungal DNA was extracted and PCR amplification was performed. The PCR products showed clear cut band of 650bp length size which is 100% similar to our first isolated fungus. Jahan *et al.* [19] reported similar results for crown rot disease of banana fruit causing fungus which support our present findings. The above results confirmed the isolated fungus as *Pichia kudriavzevii* which is responsible for brown rot disease of *Citrus sinensis* fruit.

In the present study, different controlling techniques were evaluated against the *P. kudriavzevii* fungus. Methanol extracts of *Allium sativum* and *Psidium guajava* showed the antifungal activity against the isolated fungus that could be used as natural controlling agent. Some medicinal plant extracts have been reported by some researches [25-27] as antifungal agents. Chowdhury *et al.* [24] reported that methanolic extracts of *Ficus racemosa*, *Moringa oleifera*, *Azadirachta indica*, *Cassia alata*, and *Senegalia catechu* have been significant antifungal activity against some selected pathogenic fungi. So, *Allium sativum* and *Psidium guajava* plant extracts can be used as natural fungicides. In the present study, we also investigated the antagonistic activities of some nonpathogenic bacteria and fungi against the *P. kudriavzevii* isolate. The nonpathogenic bacteria, *Rhizobium phaseoli*, *Rhizobium leguminosarum* and fungi, *Colletotrichum gloeosporioides* and *Alternaria spp.* showed significant antagonistic activity against the isolated fungus that could be used as inhibitory agent against the isolate *Pichia kudriavzevii*.

Thus this study provided information regarding the prevalence and the alternative for chemical fungicide for the isolate from brown rot disease of *Citrus sinensis*. The present data will help in agriculture sector to provide appropriate natural fungicide to controlling the brown rot disease of citrus fruits in postharvest condition.

Conclusion

The present findings revealed the detailed of isolation, characterization and biological control management of the causal agent of brown rot disease of *Citrus sinensis* by eco-friendly. To

control any pathogen, it is very important criteria to know its identity and characteristics. Hence, the morpho-physiological and molecular detection of *Pichia kudriavzevii* was performed which is responsible for brown rot disease of *C. sinensis*. We can detect and classify the isolated fungal precisely and rapidly using the DNA-based technology. The usefulness of ITS sequencing has already been proved in phylogenic analysis of the fungal pathogenic strains isolated from the surface of sweet orange according to the present investigation. Different biological control techniques were studied against the isolated fungal stain. Some antagonistic agents and medicinal plant extracts showed efficient inhibition of *P. kudriavzevii* which is responsible for brown rot disease of *Citrus sinensis*. Our findings will be helpful for rapid detection and to further study the pathogenesis, molecular evolution and to control this devastating fungal pathogenic disease.

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Competing interests

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Authors' contributions

This work was carried out in collaboration among all authors. Author MFH, MAI and BS designed the study and wrote the first draft of the manuscript. Author MFH collected the data and carried out analysis. MAI and BS assisted with manuscript preparation. All authors read and approved the final manuscript.

Consent and ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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