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Isolation and Molecular Characterization of a Wild Type *B. cinerea* from Infected Bottle Gourd (*Lagenaria sicerari*) in China

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Authors' contributions

This work was carried out in collaboration with all authors. Author MK designed the study, carried out isolation and characterization of B. cinerea, wrote the protocol, molecular cloning, sequences analysis and wrote the first draft of the manuscript. Authors AAB and MOF reviewed the draft. All authors read and approved the final manuscript.

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ABSTRACT

Botrytis cinerea is an important phytopathogenic fungus that causes diseases in wide range of hosts, including various fruits, vegetables and ornamental plants. In this study, infected bottle gourd was collected from the commercial farm of Wuhan, China to characterize the pathogen. Morphological identification was done based on colony characteristics, shape and size of conidia, conidiophores and formation of sclerotia. Results showed that conidiophores arise straight from the mycelia with length of 2-8 µm × 220-480 µm, an average conidial dimension of 5-8.5 µm × 9-13 µm,

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and a size of the sclerotia ranging from 0.7-4.5 mm × 1 – 8.5 mm. Species specific primers, Bc-f /Bc-r and Bc_{108}^{+}/Bc_{563}^{-} produced 354 and 450 bp fragments, respectively. Sequence of PCR amplification of the rDNA- ITS region produced a 547 bp fragment using the universal primer pair ITS1/ITS4. In the phylogenetic tree based on ITS nucleotide sequences, the representative isolate was located within a clade comprising reference isolates of *B. cinerea*. Strain XT5-2 germ tubes formed infection cushion on heat-killed onion epidermis and successfully entered into the epidermal cells. In this study, it was found that *B. cinerea* grew well at 20℃ rather than below 15℃ and above 25℃, respectively. Pathogenicity assays showed *B. cinerea* strain was virulent and displayed water-soaked lesions after 3 days. Based on morphological symptoms, mycological features, molecular analysis and pathogenicity bio assay, this fungus was identified as *B. cinerea*. To the best of our knowledge, this is the first study on bottle gourd (*Lagenaria siceraria* (Molina) Standl.) as a new host of *B. cinerea* in China.

Keywords: Botrytis cinerea; gray mold; bottle gourd; 5.8S rDNA; RT PCR.

1. INTRODUCTION

Bottle gourd (*Lagenaria siceraria* (Molina) Standl) is an important tropical and subtropical vegetable belonging the family Cucurbitaceae. It is also called Calabash gourd, white-flowered gourd, trumpet gourd or long squash. Commonly the immature bottle gourds are used as vegetable for humans as highly nutritional value as well as medicinal values [1]. Because of the presence of high dietary fiber, zero fat and low cholesterol, it has potentiality to control obesity [2]. Its juice can reduce blood pressure due to the presence of high potassium content [3].

Botrytis cinerea Pers. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is an air borne, ubiquitous filamentous phytopathogenic typical necrotrophic fungi. *Botrytis cinerea* causes gray mold on a wide host range (about 1307 species) including many economically important vegetables, fruits, ornamental flowers and greenhouse plants [4,5]. Under the moist conditions and temperatures at 10-30℃, the conidia germinate in the existence of exogenous nutrients from senescent plant tissues (petals), pollen grain or bee honeydew [6]. Furthermore, it uses an arsenal of toxins to assist infection and colonization of host tissues [7]. *Botrytis cinerea* is continuously reported on new hosts including cucurbit and legume crops [8-10]. Recently, gray mold of bottle gourd affected by *B. cinerea* has been observed at different areas in China.

Considering these facts, this study was undertaken to confirm the causal agent of the disease and its pathogenicity by means of morphological and molecular characterization.

2. MATERIALS AND METHODS

2.1 Fungal Strain and Cultural Condition

In August 2016, some infected bottle gourds were exhibited similar symptoms of gray mold within the Wuhan of Hubei Province, China. Infected areas of the bottle gourd were surrounded by prolific gray conidia with abundant mycelial growth. Infected tissues were removed from the edge of the lesion and made into 2-mm pieces, then sterilized by 3% NaClO for 2 minutes and dipped in sterilized distilled water three times. The pieces were kept in petri dishes containing potato dextrose agar (PDA: Difco, Detroit, MI, USA) medium and incubated at 20℃ for 10 days. Single spore cultures were performed using the dilution technique. A typical isolate named XT5-2 was selected for this study. The isolates were maintained on PDA slants at 4℃ and in 30% glycerol at - 82℃.

2.2 Morphological Identification

Morphological identification was performed based on the colony characteristics and conidial morphology according to the existing literature [11]. In order to produce the conidia and sclerotia, *Botrytis cinerea* isolates were grown in 90 mm Petri dishes containing PDA for 7 days at 20℃ under both complete light and dark condition. Length and width of 100 randomly selected conidia and 20 sclerotia from each isolate were measured. Cultural characteristics such as colony color, mycelial growth habit, hyphal tip and shape of the colony were also examined.

2.3 DNA Extraction

For genomic DNA extraction, mycelial plugs of strain XT5-2 were placed into PDA (potato

dextrose agar) plate overlaid with cellophane membranes and cultured for 5 days at 20℃. The mycelium was collected and grounded to a fine powder in the presence of liquid nitrogen by the mortar-pestle. Genomic DNA was extracted by using the previously described CTAB method [12]. Extracted DNA was stored at -20℃ for further use.

2.4 Polymerase Chain Reaction (PCR) Amplification and Sequencing

The *B. cinerea* isolate was identified based on the DNA sequences of the internal transcribed spacer (ITS) region (ITS1-5.8S rDNA-ITS2) of the ribosomal DNA. Genomic DNA was used as template to amplify the ITS region using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′)/ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [13]. The resulting PCR product was separated by electrophoresis on a 1% agarose gel in 0.5x TBE buffer and visualized by staining with ethidium bromide [14]. Purified PCR product was cloned in *Escherichia coli* DHα using the vector pMD18-T (Takara, Japan) and sequenced [15].

The PCR amplification of specific fragments was carried out using two species-specific primer pairs, Bc-f (5'- GGAAACACTTTTGGGGATA-3')/Bc-r (5'-GAGGGACAAGAAAATCGACTAA-3') and Bc_{108}^{+} (5'-ACCCGCACCTAATTCGTCAAC-3')/Bc_{563}^{-}(5'-GGGTCTTCGATACGGGAGAA-3') [8,16].

2.5 Sequence Analysis

Obtained sequence was submitted to Gen-Bank (http://www.ncbi.nlm.nih.gov) to get the accession number. Homology searches were conducted using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) searched in the NCBI (National Center for Biotechnology Information) database. A phylogenetic tree was constructed based on sequence alignment using the neighbor-joining (NJ) method [17], with a bootstrap value at 1000 in MEGA 7 software [18].

2.6 Formation of Infection Cushions

For infection cushion formation assays, fresh onion epidermis washed with sterile dd H_2O and incubated at 80℃ for 1 h. Mycelial plugs taken from the periphery of a 2 days old colony were placed on heat-killed onion epidermis cell layers [19]. *B. cinerea* germ tubes formed infection cushion and penetrated into the cells. Infection cushion formation on glass slides occurred within 24 h. The infection structures were observed at 40 X magnification using an Olympus IX-71 microscope (Tokyo, Japan).

2.7 Examination of the Most Suitable Growth Temperature

The 5 mm sized 2 days old mycelium plugs were inoculated onto the center of PDA plates and were incubated at 15, 20, 25, 30 and 35℃ for 1 week with 3 independent replications [20]. The most suitable growth temperature was determined by measuring the growth rate of the colony on PDA.

2.8 Pathogenicity Tests

Pathogenicity of the investigated *B. cinerea* strain XT5-2 was tested on detached bottle gourd and leaves of 30 days old tobacco plant. Mycelial agar plugs (5 mm) were removed from the margins of 2 days old colony using a sterilized cork borer. Agar plug was inoculated on the epidermal layer of a bottle gourd and on the tissue area beside the main vein with the mycelial side of each plug facing the leaf surface. Inoculated with sterile PDA plugs (water agar) were used as a control [21]. All bottle gourds and plants were placed on a tray which was sealed with clear plastic films to maintain high humid conditions and incubated in a growth chamber at 20℃ with a 12 h photoperiod. Lesion diameter around each inoculated agar plugs were measured after 3 days after inoculation (DAI). This experiment was repeated twice.

2.9 Data Analysis

Experimental data (radial growth rate and lesion diameter) were subjected to analysis of variance (ANOVA) using SAS®8.0 (SAS Institute, Cary, NC, USA) program. Treatment means were compared with least significant difference (LSD) test at t = 0.05 level.

3. RESULTS

3.1 Cultural and Morphological Characterization

Collected bottle gourd was severely damaged and covered by grayish arterial mycelia with prolific spores (Fig. 1 A, B). Spores were collected and placed on PDA medium for obtaining single spore colony. Initially whitish fluffy aerial branched, septate, hyaline to brown,

compact or radial pattern mycelia were observed in PDA medium at 20℃ but finally turned into light grey or brown or dark grey color with abundant conidia and sclerotia nearly 10 to 12 days apart. After 15 days of incubation numerous randomly dispersed round or irregular shaped sclerotia ranging from 0.7-4.5 mm × 1 – 8.5 mm were seen onto the PDA medium (Fig. 2 A, B, C). Numerous hyphal tips were observed under microscopic observation (Fig. 3 A). Conidiophores (2-8 µm × 220-480 µm) arise straight from the mycelia which were branched at the top (Fig. 3 B).

Conidium was single celled, circular or elliptical, nearly obtuse or slightly tipped at the top, ovoid to ellipsoid or grape shaped and the average length was (5-8.5 µm × 9-13 µm). Mature conidia were colorless or pale brown to dark brown (Fig. 3 C).

3.2 Molecular Characterization

B. cinerea identification was done by PCR molecular technique by using total three pairs of primers. The two *B. cinerea* species specific primer sets, Bc-f /Bc-r and Bc_{108}^{+}/Bc_{563}^{-} produced 354-bp and 450-bp fragments, respectively (Fig. 4). Compared with known sequence of *Botrytis cinerea*, both specific PCR products displayed 99 to 100% sequence identity. Sequence of PCR amplification of the rDNA- internal transcribed spacers (ITS) region and 5.8s ribosomal DNA (5.8S rDNA) produced a 547 bp fragment using the universal primer pair ITS1/ITS4. The ITS-5.8s rDNA nucleotide sequences for the isolated fungi was determined and deposited in the NCBI database (GenBank accession number **KX721051**). Blast analysis confirmed the identity of the experimental fungus showing 99-100% similarity to the ITS- 5.8s rDNA sequence of publicly available published *B. cinerea* isolates. Phylogenetic tree based on ITS nucleotide sequences showed that representative isolate and reference isolates of *B. cinerea* were located in the same clade (Fig. 5).

3.3 Formation of Infection Cushion

B. cinerea strain XT5-2 germ tubes formed appressoria like structure on heat-killed onion epidermis and entered into the epidermal cells (Fig. 6). Infection cushions are hyphal aggregates that characterize alternative penetration structures [22]. Infection cushion formation was initiated within 6 to 24 h.

3.4 The Most Suitable Growth Temperature

Temperature has crucial effect on the fungal growth, sporulation and sclerotia production. It was observed that *B. cinerea* strain XT5-2 grow well at 20℃ compared to other experimental temperature. No growth occurred at 35℃ (Fig. 7).

3.5 Pathogenicity Test

The *in vivo* assay with mycelial plug inoculation was done onto the tobacco leaves All inoculated leaves displayed water-soaked lesions after 3 days (Fig. 8). *B. cinerea* was recovered from the lesions and inoculated on the healthy bottle gourd (average lesion size 3.10 cm, 20℃, 96 h) to confirm Koch's postulates and its identity was reconfirmed by morphological and molecular analyses (Fig. 9). No symptoms were observed on the control leaves.

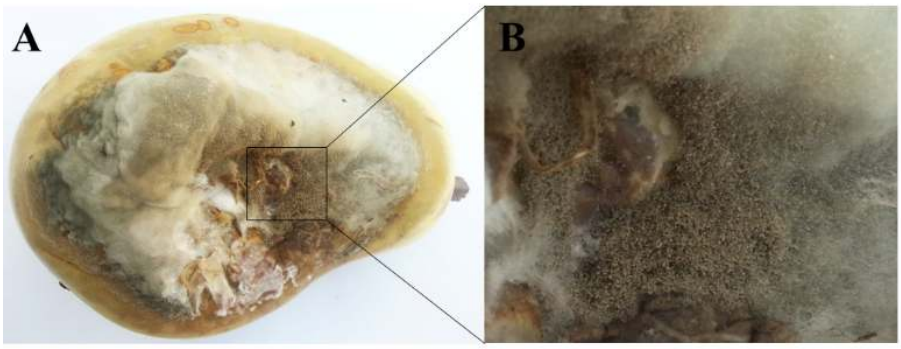

Fig. 1. Symptoms caused by *Botrytis cinerea* on bottle gourd (*Lagenaria siceraria* (Molina) Standl); (A). Whole bottle gourd damaged by *Botrytis cinerea*. (B) Close view shows prolific conidia

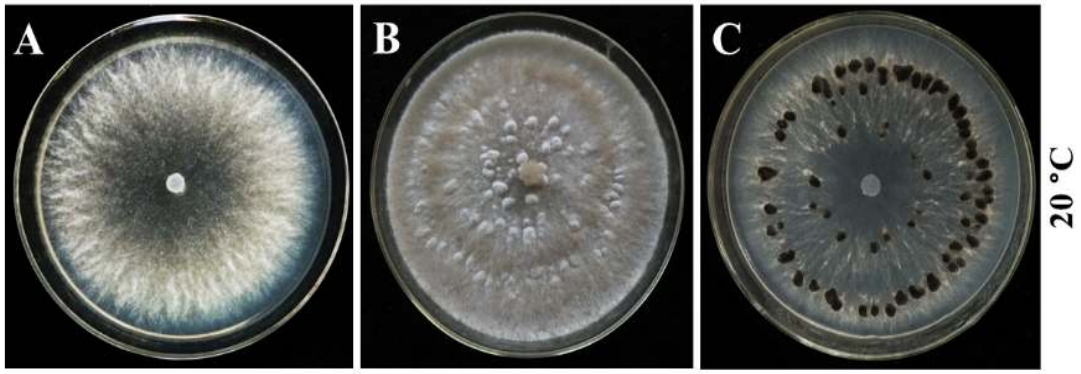

Fig. 2. Morphological characteristics of *Botrytis cinerea* on PDA. (A) Four days old colony; (B) Ten days old colony; (C) Twenty days old colony

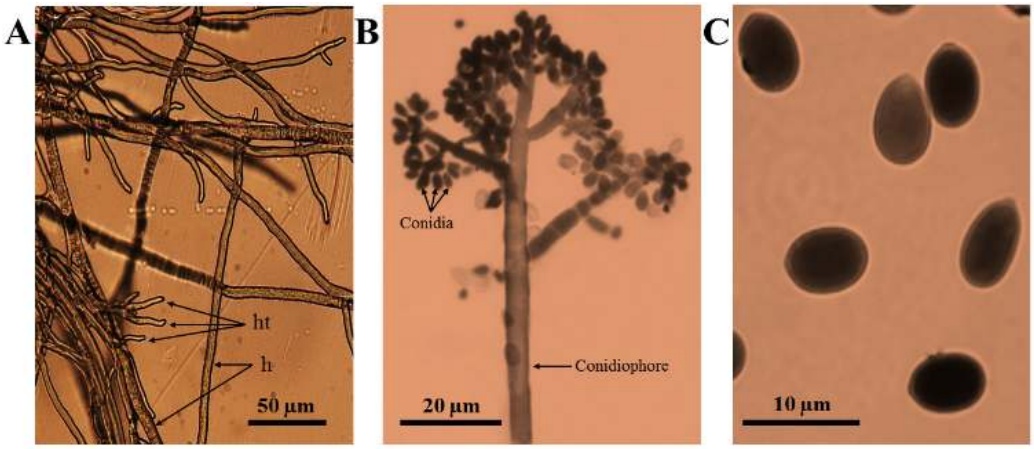

Fig. 3. Microscopic observation of different structures of *B. cinerea*. (A) Mycelial structures of *B. cinerea*; (B) Conidiophore of *B. cinerea* (bar = 20 µm); (C) conidia of *B. cinerea* (bar = 10 µm)

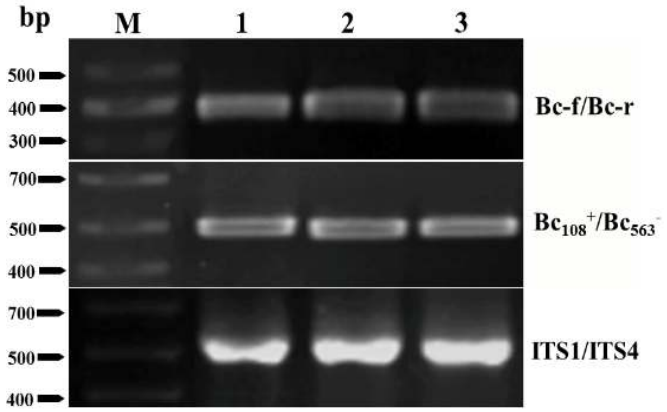

Fig. 4. PCR products of *Botrytis cinerea* isolate amplified with two species-specific primer pairs (Bc-f/Bc-r and Bc_{108}^{+} /Bc_{563}^{-}) and primers for the amplification of internal transcribed spacer (ITS) region (ITS1 and ITS4). Lane M: DL 1000 DNA marker; lanes 1-3: replications of the *Botrytis cinerea* strain XT5-2

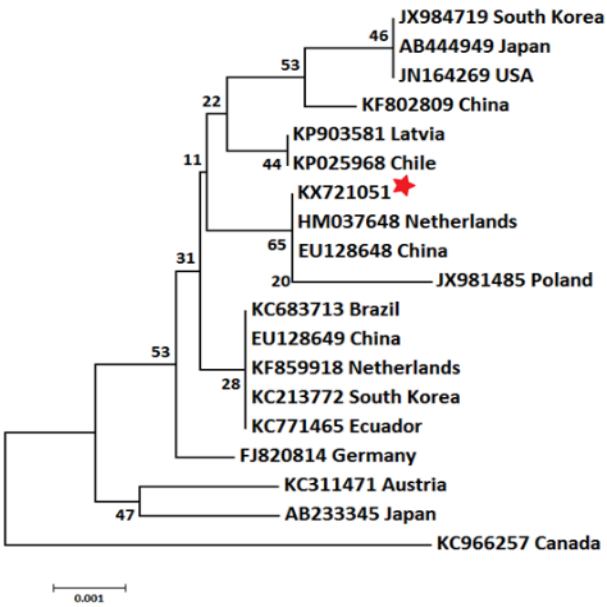

Fig. 5. Phylogenetic analysis of *Botrytis cinerea* isolate XT5-2, constructed by using the neighbor-joining (NJ) method based on the representative submitted ITS sequence. The red star shows the position of *Botrytis cinerea* strain XT5-2. The other taxa are indicated with GenBank accession numbers, followed by the areas of origin. Bootstrap numbers out of 1000 replicates are given on the nodes. The bar represents substitutions per site

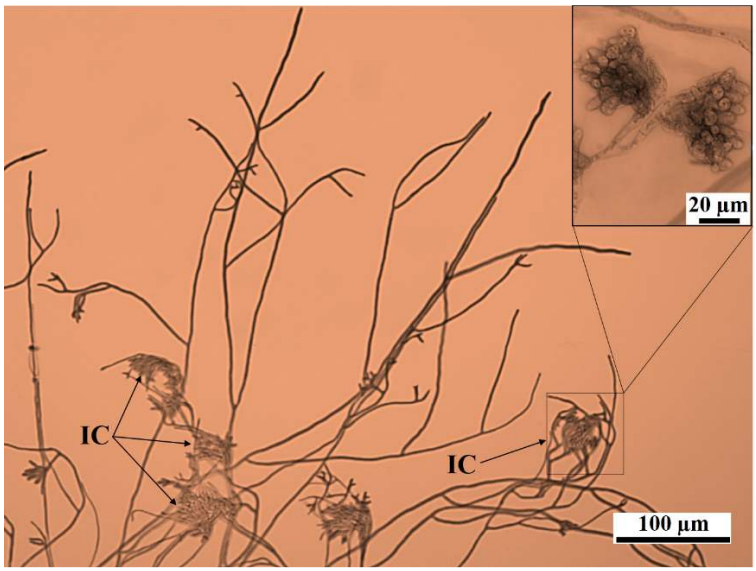

Fig. 6. Infection cushion (IC) formation by *Botrytis cinerea* XT5-2 on dead onion epidermis after 24 hrs at 20℃. Arrows indicates the IC

4. DISCUSSION

Traditionally identity confirmation of Botrytis species is largely based on the morphological characteristics, such as morphology of colonies, shape and size of conidia, conidiophores and formation of sclerotia or ascospores. According to the morphological characteristics and pathogenicity assay, tested fungus was identified as *B. cinerea* [11,23]. Morphological characteristics and biological features are frequently used to detect both among different

Botrytis species and among strains within species [24-26]. However, growth pattern, size and shape of sclerotia of *B. cinerea* is highly depend on environmental condition [27,28]. In this study, strain XT5-2 produced slightly different sizes of conidiophores, conidia and sclerotia compared with other reports [29-31]. This morphological variation maybe the reason of environmental difference and/or host specifies. In this circumstance, molecular identification was performed to confirm the identity of *B. cinerea* strain XT5-2. For molecular assays, particularly two pairs of species specific primers were used which amplified same band suggested that identified strain was *B. cinerea* [8,16,32]. Moreover, it is also confirmed by the ITS sequence that showed 100% similarity with reference sequence of *B. cinerea* in GenBank. Hoist-Jensen et Al. [33] reported that ITS sequences are useful in separating *Botrytis* from the other genera in *Sclerotiniaceae*. Phylogenetic analysis of the ITS sequence indicates that strain XT5-2 represents *B. cinerea* [17]. Author [34] reported that formation of infection cushions in infecting leaves of oilseed rape (*Brassica napus*) and tomato (*Lycopersicon esculentum*) is an important infect characteristics for *B. cinerea*. Ability of the formation of infection cushion is highly variable in intra species *of B. cinerea* [7,35]. In this study, it was found that the growth rate of *B. cinerea* was impressive at 20℃ rather than below 15℃ and above 25℃, respectively. Similar finding was reported by others [35,36].

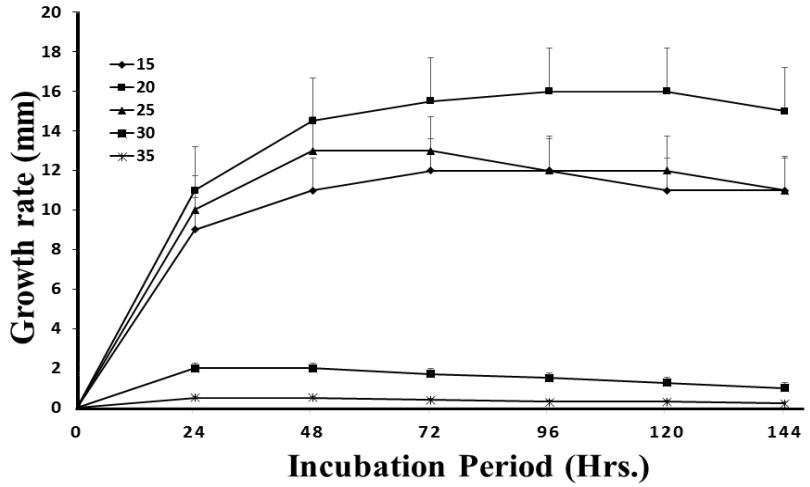

Fig. 7. Effect of temperature on the growth rate of *Botrytis cinerea* XT5-2. The data are presented as the mean value ± sd

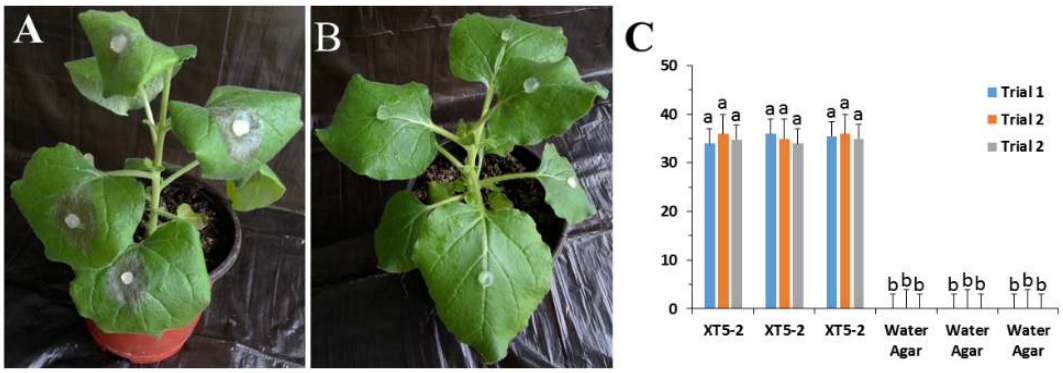

Fig. 8. Pathogenicity assay on live tobacco plant (20℃, 3 DAI). (A) Disease lesion on live tobacco leaves. (B) Control inoculation with agar plug without mycelia. (C) lesion diameter (mm) after 3 DAI. The data are presented as the mean value ± sd

Fig. 9. Koch's postulates on detached bottle gourd (20 ℃, 4 DAI). Clear disease lesion on bottle gourd but Control inoculation with agar plug failed to formation any disease lesion

The results of pathogenicity assays confirmed that, strain XT5-2 is able to cause disease lesion. It was already reported that, *B. cinerea* causes gray mold diseases on a vast host range including legume and cucurbits hosts [37-39]. Nevertheless, there have been no reports on bottle gourd.

5. CONCLUSION

Based on morphological symptoms, mycological features, molecular analysis and pathogenicity bio assay, this fungus was identified as *B. cinerea*. Gray mold disease of bottle gourd (*Lagenaria siceraria* (Molina) Standl.) caused by *B. cinerea* has not been reported. Hence, this is the first study of bottle gourd as a new host of *B. cinerea* in China. This result indicates that *B. cinerea* might be a new risk to commercial bottle gourd production. Affected tissues or vines of bottle gourd could also assist as a primary inoculum of other economic important crops.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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