**Characterization and sensitivity of *Botrytis cinerea* to Benzimidazole and SDHI fungicides and illustration of resistance profile**

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**Abstract**

*Botrytis cinerea* causinggray mold in *Solanum Lycopersicum* Mill., is one of the most destructive ascomycete pathogen, leads to sever pre and post-harvest yield losses throughout the world. SDHI and benzimidazole are broad-spectrum group of fungicides, used to control the fungal pathogenic diseases of plants. Resistance in pathogen towards these fungicides is conferred by point mutations. In our study, we study the isolate, identify and study the mechanism of resistance in *B. cinerea* by developing *in vitro* fungicides assay and fitness studies. All five isolates identifies as *B. cinerea* and cause pathogenicity on fruits. Further, isolates showed phenotypic resistance profile and multiple fungicides resistance (BosR FluR CarMR), (BosR FluMR CarMR), (BosMR FluR CarR), (BosMR FluMR CarMR) (BosS FluS CarS) was observed and EC50 (µg/ml) of boscalid-resistant isolates ranges “1.10-3.51”, fluopyram-resistant ranges “1.05-2.25” and carbendazim-resistant isolates ranges “0.89-3.82”. To investigate the molecular mechanism of resistance, *Sdh*-B gene of SDHI-resistant and *β-tubulin* gene of carbendazim-resistant isolates was amplified and amino acid sequence of resistant isolates compared with the sequences of sensitive. SDHI-resistance isolates of *B. cinerea* showed the change in amino acid sequences at codon position 272 as mutation “H272R” while carbendazim-resistant isolates showed change in amino acid sequence at codon position 198 as mutation “E198K”. *In vitro* fitness assays illustrated that *B. cinerea* produce abundant spores and the conidia germination of all isolates was also significant. At optimum temperature, higher mycelial growth was observed that was completely inhibited at 37°C. Further, *in vivo* results showed that *B. cinerea* isolates were aggressive to cause pathogenicity. The presence multiple fungicides resistance of *B. cinerea* in kingdom must be monitored constantly. Integrated disease management and application of new low resistance risk fungicides should be implemented for the reduction of future resistance risk in *B. cinerea* in this region.

**Keywords**: Gray mold; *Botrytis cinerea*; Multiple fungicide resistance; Molecular resistance mechanism; Mutations

**Introduction**

Tomato (*Solanum Lycopersicum* Mill.) is the most significant horticultural crop which is widely cultivated and consumed throughout the world (Opeña and Van der Vossen, 1993). It is also grown in the Kingdom of Saudi Arabia at large scale. In 2019, the total harvested area was 13055 and yield of fresh fruits of tomato 254774 hg/ha, and production was about 306389 tonnes (FAOSTAT, 2019). Optimum temperature and humidity are the key factors which causes pre and post-harvest crop vulnerability towards different fungal, bacterial, viral pathogen infections resulting significant yield reduction (Kumari *et al.,* 2014). Gray mold disease also known as “Botrytis fruit rot” is one of most destructive disease of tomatoes and strawberries caused by *Botrytis cinerea*, prevalent in all tomato-growing regions globally under humid and cool conditions and is the most destructive that causes serious and significant yield losses worldwide (Zhang *et al.,* 2020). It appears on healthy tomatoes even during storage or transportation and the outlay damage by *B. cinerea* is hard to conclude, as it appears at numerous production stages and distribution chain (Dean *et al.,* 2012).

Integrated management strategies like resistant cultivars and reduction of inoculum reduction are considered the best approaches for the control of gray mold. However, application of chemicals still is the key approaches for the management of *B. cinerea* (Fan *et al.,* 2017; Yin *et al.,* 2018). Due to the genetic diversity and substantial spore production of *B. cinerea* it is difficult to control this disease (Leroux *et al.,* 2002). However numerous fungicides with different mode of action have been used to control such disease. Once, benzimidazole fungicides effectively control the gray mold, but the potency of fungicides after long-term applications declined rapidly due to pathogen resistance (Han *et al.,* 2011; Su *et al.,* 2019). In 2000, Rosslenbroich and Stuebler, recognize five categories of fungicides based on their mode of action including, Microtubule assembly, Osmoregulation, Respiration affecting, Sterol Biosynthesis Inhibitors, whose toxicity reversed by amino acids. With the decline in efficacy of these fungicides, new fungicides with different modes of action (MOA) were developed (Leroux and Walker, 2013). For the management of gray mold, various site specific fungicides like, methyl benzimidazole carbamates (MBCs), succinate dehydrogenase inhibitors (SDHIs), quinone outside inhibitors (QoIs), anilinopyrimidines (APs), dicarboximides (DCFs) and phenylpyrroles (PPs) are available which are widely used because application of chemicals is considered the most quick and efficient way to control this disease. Numerous treatments in a specific period ranges from one or two, to more than twenty and particularly, after fruit harvesting, seeds or bulbs treatment with fungicides are helpful (Leroux, 2004). But over the last 30 years, resistance of *B. cinerea* to multiple fungicides has widely been reported after their introduction for the control of gray mold (Moyano *et al.,* 2004; Myresiotis *et al.,* 2007; Yin *et al.,* 2018). Numerous studies has reported the resistance in *B. cinerea* against multiple fungicides (Zhang *et al.,* 2009; Fernández-Ortuño *et al.,* 2012; Chen *et al.,* 2016). Currently, frequent use of SDHI and QoI fungicides resulted the resistance development in *B. cinerea* (Bardas *et al.,* 2010; Samaras *et al.,* 2016).

Due to resistance development in *B. cinerea*, Fungicide Resistance Action Committee (FRAC) has reported this pathogen at high-risk (https://www.frac.info/).

Now, SDHI and benzimidazole fungicides have very high risk of resistance against *B. cinerea*. Molecules of this class are generally introduced into the group of botryticides. SDHI fungicides (carboxin and flutolanil) were initially developed for action against basidiomycetes but over last 10 years, the developed subsequent generation of molecules has a superior rising activity spectrum to ascomycetes *i.e* *B. cinerea*. The target of SDHI fungicides is succinate dehydrogenase (SDH) that is, positioned inner part of mitochondrial membrane of eukaryotes (Matsson and Hederstedt, 2001). The target of SDHI is mitochondrial complex II enzyme that plays a major role in mitochondrial electron transport chain and tricarboxylic acid cycle and composite of 4 proteins: *Sdh-*A, *Sdh-*B, *Sdh-*C and *Sdh-*D while *Sdh-*B, *Sdh-*C and *Sdh-*D subunits have ubiquinone binding sites (Hagerhall, 1997). SDHI restrain respiration of fungi by blocking of this site within the ubiquinone receptacle. They have a configuration with an integral carboxamides group (Horsefield *et al.,* 2006).

Numerous studies reported a moderate to high-level resistance risk in SDHI and benzimidazole fungicides (Bardas *et al.,* 2010; Miyamoto *et al.,* 2010; Avenot *et al.,* 2012). After the introduction of boscalid for the control of deuteromycetes and ascomycetes, resistance towards SDHIs increased sharply (Avenot and Michailides, 2007; Leroux *et al.,* 2010). Resistance to SDHI’s is mediated by single point mutation that leads to substitution of amino acid at subunits: *Sdh*-B, *Sdh*-C and *Sdh*-D (Ito *et al.,* 2004; Avenot *et al.,* 2008; Avenot *et al.,* 2009; Miyamoto *et al.,* 2010) while benzimidazole resistance has been reported with the point mutation of glutamic acid (GAG) to glycine (GGG) substitution at the same position 198 (E198G) that are identified in a highly benzimidazole-resistant laboratory mutant strain but molecular analysis of the moderately benzimidazole-resistant strains revealed no mutations at these positions in the *β-tubulin* gene (Elad *et al.,* 2004; Yin *et al.,* 2011). Reported resistance in *B. cinerea* strains or laboratory mutants exposed eight assorted amino acid variations in subunits *Sdh*-B or *Sdh*-D related to SDHI’s resistance (De Miccolis *et al.,* 2010; Leroux *et al.,* 2010; Veloukas *et al.,* 2011). Substitutions of arginine H272R at codon 272 of *Sdh*-B or histidine by tyrosine H272Y were recurrent mutations discovered in field strains. The third mutation at the similar codon, by surrogating histidine with leucine H272L, has also been found at small frequencies (Veloukas *et al.,* 2011; Leroux *et al.,* 2010). A different mutation in *Sdh*-D subunit, leading histidine substitution by arginine at codon 132 H132R has also been reported (Leroux *et al.,* 2010).

For the control of gray mold disease, farmers use the combination of fungicides or exchanged fungicides with different modes of actions that lead to the development of resistance. Frequent uses of fungicides have developed the resistance in *B. cinerea*, which become a serious challenge and to reduce the risk of resistance development, new strategies are needed to single and multiple fungicides. However, *B. cinerea* has also been reported in seed born mycoflora in the kingdom of Saudi Arabia (Al-Askar *et al.,* 2014) which is the most serious and challenging pre and post-harvest disease. To the author’s knowledge, no further study has been conducted for the management of *B. cinerea* but based on reported resistance problems worldwide, present study was proposed to: (i) isolate and identify the *B. cinerea*; (ii) to study the sensitivity of *B. cinerea* to fungicides in this region (iii) to study the mechanism of fungicide sensitive/resistance in *B. cinerea*. The outcome of this study will assist to know and assess the current status of *B. cinerea* in this region.

**Materials and Methods**

**Isolation and culture condition**

In 2019, samples of infected fruits of tomato were collected from Agriculture Research Station (Hada Al-Sham) of King Abdulaziz University, Saudia Arabia. Small pieces of diseased tomato leaves were surface sterilized with 2% NaOCl for 2-3 min and rinsed thrice in distilled water. After drying, pieces were directly transferred to PDA (200g potato; 15g Agar; 18g Glucose; 1L water) medium plates. Infected tomato fruits were collected from vegetable market of Jeddah. Single spore transfer technique was used for the isolation from infected fruits. Sterile cotton swabs were slightly abrased on the infected surface of tomato fruits to collect the spores and touched on the surface of PDA plates. Plates were incubated at 17˚C in darkness for colony growth. Morphological characteristics *i.e* conidia size, color and hyphal growth was observed and 5 isolates of *Botrytis cinerea* wereisolated. *B. cinerea* colonies were purified by transferring a small fungal disc from the edge of 3-days old colonies to new PDA plates. Colonies were preserved and maintained on PDA medium glass tubes containing glycerol and tubes were incubated at 4˚C.

**Identification of *Botrytis cinerea***

**Koch’s experiment**

Pathogenicity of all isolates wasconfirmed by Koch’s postulates. Further, Isolates were grown on PDA plates. Tomato fruits of the same size were purchased, surface sterilized in 2% NaOCl for 5 min, washed thrice with distilled water. From three days old colonies, 5-mm mycelial disc removed from the margin was placed on tomato fruits, artificially injured with sterilized needle. Agar plug lacking mycelium was placed on tomato fruits as control. Fruits were placed in sterilized plastic boxes containing three layers sterile filter paper to retain moisture. Boxes were incubated at 17˚C for 3 days and symptoms were observed on fruits, lesion diameter was measure. Germinated colonies were re-isolated and compared with those, isolated first time. Three fruits were used for each isolate. Experiment was conducted twice and results were compared.

**Molecular Identification of *B. cinerea***

For molecular identification of *B. cinerea* isolates, genomic DNA was extracted by cetyltrimethyl ammonium bromide (CTAB) method (Mafra *et al.,* 2008) with slight modification. Isolates were grown on PDA plate and 5mg mycelium was collected in 2 mL microcentrifuge sterile tubes carrying 1 mL of CTAB extraction buffer (CTAB 20 gL–L; NaCl 2 molL–1; Tris 0.2 mol L–1; EDTA 5 mmol L–1). Buffer was mixed and placed in water bath at 65°C for 30 min. Then, 650μL of phenol-chloroform-isoamyl alcohol (25:24:1v/v) was added and tubes were centrifuged at 12,000 rpm for 10 min. After that, 650 μL supernatant was transferred to new sterile tube, mixed with chloroform-isoamyl alcohol (24:1) and centrifuged at 12,000 rpm for 10 min. Then, 600 μL supernatant was mixed with 360 μL of isoamyl alcohol and placed at -20°C for 2 hours. Tubes were again centrifuged at 12,000 rpm for 10 min and extraction was washed with 800μL ethanol (85% v/v) solution. After washing, tubes were centrifuged at 7,500 rpm for 5 min and supernatant was discarded. Pallet was dried and 50μL sterilized deionized water was added. DNA quantity was observed at NanoDrop 2000C and DNA was stored at -80°C.

**Polymerase china reaction (PCR) analysis**

For identification, genomic DNA extracted previously, was used and gene fragments of ITS region were amplified to detect the encoded protein by PCR. PCR reaction was performed with Taq DNA polymerase and by using specific primer pair (Zhang *et al.,* 2020). ITS1+ (TCCGTAGGTGAACCTGCGG), ITS4+ (TCCTCCGCTTATTGATATGC) with the final volume of mixture 25μL containing 1.5μL DNA template, 0.5μL of forward primer and 0.5μL of reverse primer, 0.3μL Taq DNA polymerase, 1.0μL dNTP, 2.5μL of 10×DNA polymerase buffer, and the rest deionized distilled water was added up-to final volume. PCR conditions were: 94 ℃ for 3 min followed by 25 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 60 s, and 72℃ for 10 min, holding the reaction at 4℃. Products of amplified genes were confirmed on 1% agarose gel. Products were sequenced (Macrogen, Seoul, South Korea) and the length of sequenced products was compared with the sequence in National Center for Biotechnology Information (NCBI) gene bank.

**Fungicides and Reagent**

Fungicides used in this study were boscalid (95%), fluopyram (98.8%), and carbendazim (98%) provided by Saudi Basic Industries Corporation (SABIC). Stock solutions were prepared by dissolving fungicides in Dimethyl sulfoxide (DMSO) and stored at 4℃ for further use.

**Fungicides assay**

Sensitivity of five *Botrytis cinerea* isolates was assessed with the reported MIC of fungicides. Briefly, *B. cinerea* isolates were grown on PDA plates for three days. Yeast Broth Agar (10g Bacterial peptone, 15g Agar, 10g Yeast Extract Powder, 20g sodium acetate and 1000 ml distilled water) medium plates were amended with the concentrations of both fungicides boscalid and fluopyram: 10 and 100 µg/mL and control (De Miccolis *et al.,* 2014) prepared from stock solution in DMSO. YG medium (5g Yeast Extract, 18g of dextrose, 15g Agar, 1000mL distilled water) plates were amended with 5 and 100 µg/mL concentrations of carbendazim fungicide (Leroux *et al.,* 2002). Dimethyl sulfoxide (DMSO) was used as control for all fungicides. Then, 5-mm mycelial disc from the margin of each isolate was removed and placed face-down on fungicides amended plates and incubated at 17˚C in darkness. After three days colony diameter was observed and isolates were categorized into highly resistant (HR) and moderate resistant (MR) and sensitive (S) as reported (Leroux *et al.,* 2002; De Miccolis *et al.,* 2014).

**EC50 of*****B. cinerea*****isolates**

**Response of*****B. cinerea*****to SDHI and benzimidazole fungicides**

Two SDHI fungicides (boscalid and fluopyram) were used with the concentrations 0.1, 1, 10, 50, and 100 µg/mL for each amended in YBA media to determine EC50 µg/mL. Concentrations of both fungicides were prepared from stock solutions of fungicides in Dimethyl sulfoxide (DMSO). Briefly, 5-mm mycelial disc from three days old *B. cinerea* culture was placed face-down on fungicides amended YBA plates. DMSO was used as control in both fungicides. Three replicates were used for each concentration including control. Plates were incubated at 17˚C in darkness until the control covers the whole plate from 3-5 days. Colony diameters were measured and EC50 µg/mL was calculated.

 For benzimidazole fungicide carbendazim, the concentrations 0.1, 1, 10, 50, and 100 µg/mL were prepared from stock solution in DMSO and these concentrations were amended in YG medium. Further, *B. cinerea* isolates were grown on PDA for three days and 5-mm mycelial disc was transferred to fungicide amended plates. DMSO was used as control and three replicates for each concentration were used. Plates were incubated at 17˚C in darkness until the growth in control completely covers the plate for 3-5 days. Colony diameter was measured and EC50 µg/mL was calculated.

**Amplification of target genes**

For partial gene “*Sdh*-B” amplification, isolates that showed resistance to both SDHI fungicides (boscalid and fluopyram) were selected along with sensitive isolates for the amplification of target gene. Using Primer pair *Sdh*-B-F (CCACTCCTCCATAATGGCTGCTCTCCGC) *Sdh*-B-R (CTCATCAAGCCCCCTCATTGATATC) (Leroux *et al.,* 2010) gene was amplified. Genomic DNA of resistant and sensitive isolates was extracted as previously mentioned. PCR fragments were amplified by using specific primers. The final volume of reaction mixture was 50µl containing 5µl (10X Standard Taq Reaction Buffer), 4µl (10 mM dNTPs), 1µl (Forward Primer),1 µl (Reverse Primer), 0.5 µl (Taq DNA Polymerase enzyme), 1 µl (Template DNA), Nuclease-free water 37.5 µl.  PCR reaction conditions were: 95°C for 3 min, followed by 35 cycles, 95°C for 30s, 60°C for 30s, 68°C for 1 min and 68°C for 4 min. PCR products were visualized by electrophoresis 1.5% agarose gel in 1×Tris-acetate (TAE) buffer staining the in 0.5μg ethidium bromide solution for 10 min.

The partial sequence of “*β*-*Tub*”gene was amplified by extracting the genomic DNA from carbendazim resistant and sensitive isolate with primer pair *β*-*tub*-F (GATCTCCAACTTGAGCGTA), *β*-*tub*-R (TGGAAACCTTACCACGGCTA) as reported (Fan *et al.,* 2017). Standard PCR reaction was performed with the conditions: 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 50.5°C for 30 s, 72°C for 1min 10 sec and 72°C for 10 min. PCR reaction was hold at 4°C . Gel was stained in 0.5μg ethidium bromide solution for 10 min and products were observed in a 1.5% agarose gel in 1×Tris–acetate (TAE) buffer and sequenced. PCR products from both genes were sequenced (Macrogen, Seoul, South Korea). Products were compared with the whole genome sequences of each gene, obtained from NCBI and possible mutations were observed in resistant isolates.

***In vitro* fitness of** ***B. cinerea*** **isolates**

**Pathogenicity on fruits**

*In vitro* pathogenicity of all isolates was determined on tomato fruits. Fruits of equal size were purchased from market, disinfected with 2% NaOCl for 5 min and rinsed thrice in distilled water. Surface of fruits was artificially wounded (3-5 punch) with sterile needle and 5-mm mycelial disk from three days old *B. cinerea* colony was placed upside downward on the surface of fruits. Agar plug lacking mycelium was placed as control. Fruits were placed in sterilized plastic boxes containing three layers of sterilized filter paper to retain moisture contents and incubated at 17°C for three days with 12h light and 12h darkness. Three fruits were used for each isolate and experiment was conducted twice. After 3 days, lesion diameter on each fruit was measured (Meng *et al.,* 2015) and mean diameter was compared to observed the virulence of pathogen.

**Sporulation**

For spore production, 5-mm mycelial disc from three days old colony was transferred to malt extract agar MEA (Malt extract 30g; agar 20g; peptone 5g; distilled water 1000ml) medium plates. Plates were incubated at 17°C for 5-7 days with 12h light and 12h darkness (Stewart and long, 1987). After 7 days, 10ml distilled water was added to each plate. Surface of germinating mycelium was scrapped and filtered through sterile cheesecloth. Suspension was centrifuged at 3000 RPM for 5 min and pallet was removed. Supernatant containing spores was collected into 5ml tubes. Spores were counted with hemacytometer under microscope and expressed as number of conidia/cm2. Three replicates were used for each isolate and spore germination was further studied.

**Germination of conidia**

Conidia germination was observed on 1.5% water agar medium plates. 1ml of spore suspension (prepared earlier) from each replicate was spreaded with sterilized glass spreader over water agar (agar 20g; distilled water 1000ml) plates. Plates were incubated for 12h at 22°C. Number of germinated conidia was counted in each plate. Conidia was said to be germinated, if germ tube was longer than conidium. Germinated conidia were expressed as percentage germination: Number of germinated conidia/total number of conidia\*100 (unpublished data). Three replicate were used for each isolate.

**Temperature sensitivity**

Isolates of *B. cinerea* were incubated at different temperatures to determine their sensitivity. Briefly, from three days old colonies, 5-mm mycelial disc was transferred to new PDA plates and plates were incubated at different temperatures, 12°C, 18°C, 25°C, 30°C, 33°C and 37°C. Three replicates were used for each isolate. After five days, colony diameter was measured and means were calculated and compared.

***In vivo* pathogenicity**

*In vivo*pathogenicity was determined on the hybrid tomato variety “DOUCEN”. Briefly, seedlings were grown in green house in pots, containing peat moss (1:3). At 3-4 leaf stages, seedlings were transferred to new pots. Plants were irrigated as per requirement. For pathogen inoculation, mycelial disc from 3-5 days old colony cultures grown at PDA was transferred to the leaves of plants. Four leaves per plant were artificially punctured and mycelial disc was placed face-down on leaves. Agar disc lacking mycelium was placed as control. For each isolate, at least four replicates were used and each replicated contained three plants. Sufficient relative humidity and temperature was maintained at 24 ± 2°C. After inoculation, plants were covered with plastic bags for 72h. Symptoms were observed and lesion diameter around each disc was calculated according to the level of aggressiveness of each isolates based on 0-4 rating scale (Munoz *et al.,* 2016). All experiments were performed in triplicates. Greenhouse experiment was conducted using completely randomized design. For statistical analysis of all recorded data, statistix 8.1 was used and one-way analysis of variance ANOVA was performed. Means of all isolates were compared using Fisher’s Least Significant Difference (LSD) test at *p*=0.05.

**Results**

**Isolation and identification of*****B. cinerea***

Five isolates were isolated from tomato fruits and leaves which were identified based on Koch’s postulates and all isolates were found pathogenic. Further, the re-isolation of isolates demonstrated that all isolates were identical to those, isolated first time. However, pathogenicity level was observed from the infected lesion area developed by each isolate. Typical symptoms of botrytis rot were observed on tomato fruits (Fig.1). Isolates were morphologically characterized based on their colony color and structure of mycelium on PDA plates. Further, molecular identification of isolates by standard PCR with specific primer pair showed 560 bp, length of these five isolates. Amplified product sequences were compared with the whole genome of *B. cinerea* obtained from NCBI (accession no NC\_037313). Blast analysis showed 99% similarity to *B. cinerea* and therefore, these isolates were confirmed as *B. cinerea*.

**Fungicide assay**

Sensitivity of *B. cinerea* was observed with previously mentioned MIC concentrations of SDHI fungicides. Further, isolates were categorized into resistant/highly resistant moderate resistance and sensitive (De Miccolis *et al.,* 2014). Out of five, two isolates were resistant and two were moderate resistant while one isolate was sensitive to boscalid while three isolates were resistant, one moderate resitant and one isolate showed sensitivity to fluopyram. From carbendazim, three isolates were moderate resistant while one resistant and one was sensitive described in Table 1. Further, resistance isolates along with sensitive, were used for amplification of target gene.

**Sensitivity to SDHI fungicides**

All isolates were further used to evaluate their EC50 µg/mL with different concentration of each fungicide mentioned previously. Boscalid resistant isolate “BC-1101 and BC1102” showed higher EC50 3.51 and 3.01 respectively while isolate BC-1103 and BC-1104 were observed with lowest EC50 2.6and 1.45 respectively as the level of resistance was low. EC50 value of sensitive isolate was 1.01. However, variation in the level of resistance was observed (Fig 2). Isolates also showed resistance to fluopyram and EC50 value of resistant isolate BC-1101and BC-1103 were 2.25 and 1.69 respectively while lower value of EC50 was observed by sensitive isolate BC-1105 as 1.05. Further, moderate resistant isolates showed EC50 values 1.76 and 1.32 by BC-1102 and BC-1104 respectively (Fig 3).

**Sensitivity to benzimidazole fungicide**

Three moderate resistant isolates, one resistant and one sensitive isolate showed the different level and variation in EC50. Moderate resistant isolates (BC-1101, BC-1102 and BC-1104) showed EC50 values 2.63, 1.07 and1.10 respectively while sensitive isolate (BC-1105) exhibited 0.89 and resistant isolate (BC-1103) showed 3.82, which was the highest as compared to other moderate resistant isolates (Fig 4).

Further, the variation in the level of resistance was found in all isolates. Isolate “BC-1101” showed resistance to multiple fungicides as it was resistant to both SDHI fungicides used in this study. However, isolate “BC-1105” was found sensitive to both SDHI and benzimidazole groups of fungicides. Based on the results, different resistant isolates were further used to study the mechanism of resistance in resistant isolates by the amplification of target gene may responsible for resistance against each fungicide.

**Sequence analysis and gene amplification**

One isolate BC-1101 was found resistant to both SDHI fungicides and BC-1105 that was found sensitive to all fungicides. The isolate was resistant to both SDHI fungicide was selected along with sensitive for amplification of *Sdh*-B responsible for resistance in *B.cinerea*. The length of amplified *Sdh*-B gene was 1000bp (Fig 5a). To find the possible single nucleotide changes sequence was compared with complete gene sequence of *B. cinerea* obtained from NCBI (accession number KR866382.1). In the partial sequence of SDHI resistant isolate “BC-1101”, a change in nucleotide sequence at codon position 272 was detected which showed the mutation from CAC to CGC change in amino acid from histidine to arginine H272R while no mutation or change in the nucleotide sequence of sensitive isolate was observed.

Isolate BC-1103 was found resistant to carbendazim while BC-1105 was sensitive. Resistant isolate along with sensitive were used for the amplification of β-tubulin gene. The length of amplified gene product was 950bp (Fig 5b). Sequences were compared with complete β-tubulin gene sequence obtained from NCBI (accession number U27198.1) and single nucleotide changes were observed. In the partial gene sequence, change in nucleotide sequence from GAG to AGG at codon 198 was detected and mutation E198K was detected in carbendazim resistant isolate while no change was observed in sensitive isolate. These changes were not found in sensitive isolates.

***In vitro* fitness**

**Pathogenicity assay**

Pathogenicity assay of *B. cinerea* isolates, on tomato fruits showed significant correlation between isolates and mean lesion diameter (mm) of all isolates was compared with control. Tough, all isolates were pathogenic and caused pathogenicity but isolate “BC-1101” and “BC-1102” were found highly virulent as lesion diameters were greater than other isolates (Fig 6).

**Sporulation and conidia germination**

*In vitro* spore production of isolate BC-1101 and BC-1102 was higher than other isolates (Fig 7). However, all isolates showed significant sporulation (cm2) either lower or in abundantly and significant correlation was observed between all isolates. The conidium of all isolates showed germination and significant relation was observed between the isolates (Fig 8a). More than 80% of conidia were germinated and the length of germinated conidia was observed (Fig 8b).

**Temperature sensitivity**

All isolates were exposed to different temperature 12°C, 18°C, 25°C, 30°C, 33°C and 37°C. However, optimum temperature was ranged from 25-30°C. Furthermore, maximum mycelial growth was observed at 25°C which slightly reduced at 30°C. When the isolates were incubated at 33°C, the mycelial growth was significantly inhibited. No mycelial growth was found at 37°C as this temperature does not allow the pathogen to grow (Fig 9). However, 12°C and 18°C temperature was also favorable but not significant which showed the initiation of pathogen while 25°C was the optimum temperature. The result of temperature sensitivity indicates that growth of *B. cinerea* pathogen requires required serious concerns to control the spread of initial inoculum at optimum temperature by applications of post harvest treatments.

***In vivo*****Pathogenicity**

The results ofpathogenicity observed in greenhouse indicated that all isolates caused pathogenicity on tomato plants. However, isolates were also pathogenic on tomato fruits as previously mentioned. Based on mean lesion diameter, isolate BC-1101 and BC-1103 were found highly aggressive as compared to others. While isolates BC-1102, BC-1104, and BC-1105 were also aggressive but the level of aggressiveness was significant (Fig 10). However, all isolates were also pathogenic.

In our results, isolate BC-1101 and BC-1102 were isolated from tomato fruits while other three were isolated from field. However, isolate BC-1101 was highly resistant to both SDHI fungicides while isolate BC-1103 was also highly resistant to SDHI and benzimidazole fungicide which clearly demonstrated the shifting of resistance in *B. cinerea* pathogen to fields.

**Discussion**

Tomato (*Lycopersicon esculentum* Mill.) is economically one of the most significant vegetable crop in Saudi Arabia. Large number of pre and post-harvest diseases has been observed on tomato but, gray mold diseases caused by *B. cinerea* is the most significant because it causes severe yield losses (Fernández-Ortuño *et al.,* 2012; Zhang *et al.,* 2020). Multiple applications of fungicides to control this disease results the resistance of pathogen and developed the combined resistance risk in *B. cinerea* (http://www.frac.info/). However, *B. cinerea* resistance, frequently compromise the fungicide efficiency and ultimately cause the failure of disease control. Resistance of *B. cinerea* to different classes of fungicides is the most threaten and challenging problem in tomato fields. Conducted survey indicates that frequent use of SDHI fungicides against gray mould disease rise severe resistance of *B. cinerea* and endangered widespread occurrence of resistance. In our study, we recovered two isolates from tomato fruits and three isolates from field. As a result of Koch’s experiments, all isolates caused the pathogenicity on fruits. Further, their molecular identification confers the post-harvest presences of pathogen on tomato fruits also in field as pre-harvest. Introduction and presence of *B. cinerea* in field has already been reported (Al-Askar *et al.,* 2014) which support our study and survey. Fungicide assay conducted with these five isolates showed different level of resistance from higher to lower. In our study, we used the reported MIC concentrations of two SDHI fungicides (boscalid and fluopyram) to study the sensitivity or resistance of *B. cinerea* isolates. Results demonstrated that two isolates were highly resistance, two showed moderate level of resistance while one isolate was sensitive to boscalid. From the results of fluopyram fungicide assay, two isolates were highly resistant, two moderate resistant and one isolate was sensitive and SDHI fungicide assay results showed that *B. cinerea* showed moderate to higher level of resistance in field *B. cinerea* isolates. Earlier, researchers reported the field resistance of *B. cinerea* in grapes fields, in France and Germany to SDHI fungicides (Leroux *et al.,* 2010; Leroch *et al.,* 2011). Resistance to in different regions has been reported i.e Germany reported in strawberries (Stammler *et al.,* 2008), Washington State (Kim *et al.,* 2010) in apple, and in Greece they reported in kiwifruit (Bardas *et al.,* 2010). Results of reported studies strongly support our findings. These studies concluded the broad host range of *B. cinerea* which is the most serious, threatening and challenging problem. Further, other than SDHI fungicide resistance, some studies reported the resistance in *B. cinerea* to Iprodione, procymidone and thiophanate-methyl (Lopes *et al.,* 2017), pyrisoxazole (Zhang *et al.,* 2020), difenoconazole (Zhang *et al.,* 2020) and cyprodinil (Fan *et al.,* 2017). In our results, In our results, EC50 (µg/ml) of boscalid resistant BC-1101, and BC-1102 was 3.51 and 3.01 respectively while moderate resistant isolates (BC-1103, BC-1104) showed 2.06 and 1.45 while sensitive isolate BC-1105 showed 1.01. However, EC50 (µg/ml) of fluopyram-resistant (BC-1101,BC-1103) was 2.25 and 1.69 respectively while moderate resistant isolates (BC-1102, BC-1104) showed 1.32 and 1.76, while sensitive isolate BC-1105 showed 1.05. Isolate “BC-1105” was sensitive to all fungicides. In our results, EC50 (µg/ml) of resistant isolates was higher than sensitive one and moderate resistant isolates showed EC50 (µg/ml) lower than resistant and sensitive isolate. Similar finding has been reported by other researchers, which support our results (Bardas *et al.,* 2010; De Miccolis *et al.,* 2014]. Gene amplification and sequence alignment of one SDHI-resistant (resistant to both SDHI fungicide) isolate showed change in nucleotide sequence at codon position 272 which showed the mutation from CAC to CGC change in amino acid from histidine to arginine H872R while no mutation or change in the nucleotide sequence of sensitive isolate was detected. Researchers from France and Germany reported three mutations (H272L, H272R, and H272Y) in SDHB from isolates of *B. cinerea* collected from strawberries or grapes (Stammler *et al.,* 2008; Leroux *et al.,* 2010) which strongly support our findings. However, SDHI-resistant isolates of *B. cinerea* showed five free mutations, positioned in SDHB subunit containing iron-sulfur protein of mitochondrial SDH (Cecchini, 2003). In some pathogenic fungi, mutations at the analogous histidine codon were formerly revealed to be the major mutations related with resistance to SDHIs. In *Corynespora cassiicola*,H278Y/R mutation was reported (Miyamoto *et al.,* 2010). Substitutions of histidine (his) by either tyrosine (tyr) or threonine (thr) at codon 277 (H277Y/R) have also been related to boscalid-resistant isolates of *A. alternata* from pistachio but, the resistance responsible for mutation to boscalid was observed at codon 134 in SDHC (H134R) in this pathogen (Avenot *et al.,* 2009). In the subunit of *Sdh*-B, position of histidine mutations corresponds to one of the two Qp sites (ubiquinone binding sites) that has been determined in the vicinity to third complex of iron-sulfur (3Fe-4S) (Sun *et al.,* 2005). In past, numerous molecular approaches have been developed for the rapid detection of resistance to fungicide *i.e* PCR, Allele-Specific (AS-PCR), Restriction Fragment Length Polymorphism (RFLP) and Pyrosequencing (Ma and Michailides, 2005). For rapid detection of resistance in the strains of *Fusarium graminearum,*PIRA-PCR has been used(Luo *et al.,* 2009) and also in the strains of *Magnaporthe grisea* resistance against capropamide (Kaku *et al.,* 2003). PIRA-PCR has also been used for quick detection of P225F, N230I and H272Y/L/R mutations found in the subunit of SDHB of *B. cinerea* boscalid-resistant isolates. In subunit of SDHB, primer pairs in PIRA-PCR properly genotyped all of the five mutated codon (Veloukas *et al.,* 2011). Evidently, the improvements of primers for additional SDH mutations that have previously reported *in vivo* or *in vitro* studies for *B. cinerea* isolates would be an important input to quicker detection of resistance.

In our study, the results of benzimidazoles fungicide (carbendazim) assay demonstrated that three isolates were moderately resistant, one isolate was resistant and one isolate was sensitive to this class of fungicides. EC50 (µg/ml) of carbendazim-resistant (BC-1103) isolate was 3.82, moderate resistant isolates (BC-1101,BC-1102, BC-1104) showed 2.6, 1.07 and 1.10 respectively while sensitive isolate (BC-1105) showed 0.89. In our results the EC50 (µg/ml) of resistant isolate was higher than sensitive and similar findings has already been reported (Sun *et al.,* 2010; He *et al.,* 2020) which strongly support our results. Gene amplification of carbendazim resistant isolate showed single nucleotide changes. In the partial gene sequence, change in nucleotide sequence from GAG to AGG at codon 198 was detected and mutation E198K was found in carbendazim resistant isolate while no change was detected in sensitive isolate in comparison with whole *β*-*tub* gene sequence. Similar mutation in carbendazim resistant isolates has been reported by many researchers from different locations (Liu *et al.,* 2016; Liu *et al.,* 2019; He *et al.,* 2020).Widespread botryticides resistance in older classes *i.e* anilinopyrimidines, dicarboximides and benzimidazoles has already been reported and dual resistance to SDHIs by pathogen and recently introduction of QoIs is also very general trait (Bardas *et al.,* 2010; Leroux *et al.,* 2010; Kim *et al.,* 2010). In *Sdh-*B, the conferred resistance level by every mutation might have significant insinuations on SDHI fungicides performance that used for gray mold (Veloukas *et al.,* 2013). A low level of control was found by boscalid and show efficiency against two mutants that acquired the mutations *i.e* P225F and H272L. A low to moderate efficacy was found in control against mutants demonstrating mutations N230I and H272Y/R (Veloukas *et al.,* 2011). In strawberries field from Greece, predominant mutation H272R was found in field population (Veloukas *et al.,* 2011). Against some fungal strains like *C. cassiicola* and *P. xanthii*, high control efficacy of fluopyram that shows histidine substitution by either arginine or tyrosine at identical codon had been observed (Ishii *et al.,* 2011). Frequently application of novel molecules could exhibit some alteration in the frequency of mutation form by choice of mutants that illustrate high or moderate resistance level *i.e* H272L, N230I, P225F. Therefore, instantaneous application of structurally diverse SDHIs or alternation may leads towards the fully cross-resistant mutant’s selections (Scalliet *et al.,* 2011).

*In vitro* fitness assays showed that all isolates caused pathogenicity on fruits. However, lesion diameter of isolate “BC-1101” and “BC-1102” were highly virulent and these were found resistant to SDHI fungicides. Further, all isolates either moderate resistant or sensitive cause pathogenicity on fruits. Similar outcomes for pathogenicity have been reported from pistachio orchards and grape vineyards (Avenot *et al.,* 2020). Sporulation and conidia germination of all isolates was higher and significant correlation was observed between all isolates. Abundant sporulation was observed and conidia germination (%) of all isolates was also significant. Studies also reported the abundant sporulation and higher conidia germination of *B. cinerea* (Cotoras *et al.,* 2009; Munoz *et al.,* 2016; Isaza *et al.,* 2019). *B. cinerea* isolates showed significant correlation towards temperature sensitivity. Temperature 25°C, 30°C was the optimum temperature for colony growth but no growth was observed at 37°C. Although, mycelial germination was observed at 12°C, 18°C but not significant but mycelial growth of all isolates was significantly higher at optimum temperature. However, many researchers reported the sensitivity of *B. cinerea* to temperature and found significant growth at optimum temperature (Fedele *et al.,* 2020; Toffolatti *et al.,* 2020) but no growth at 37°C (Meng *et al.,* 2015) which resolutely support and confers our results. The results of *in vivo* pathogenicity showed that *B. cinerea* is an aggressive pathogen. In our results, isolate BC-1101 and BC-1103 were most aggressive as compared to others. However, all isolates caused significant pathogenicity in greenhouse. Aggressiveness of *B. cinerea* in greenhouse has also been reported (Liu *et al.,* 2016; Munoz *et al.,* 2016).In populations of *B. cinerea,* the resistance development to SDHIs can cause considerable troubles for management of disease because *Sdh* mutations might affect very strongly to the performance of fungicides against mutated strains. These mutations may not confer resistance. The superior consideration of those aspects that might influence the determination of SDHI resistance in *B. cinerea*, for example the effect of every specific mutation on strain’s fitness or in field applications, effect of spray programs of fungicide on every mutant selection, must be further studied. Our findings may also propose that resistance development to SDHI in the population of *B. cinerea* is not randomly and several specific genetic groups may develop resistance in populations.

**Conclusion**

In conclusion, growers can no longer rely on the efficiency of SDHI and benzimidazole fungicides to control gray mold. Particular attention is needed for the control of gray mold disease by integrated disease management strategies and must focusing the development of low risk fungicides with different mode of actions to overcome the serious and challenging problem of resistance. However, moderate resistance may exist which might be control with repeated application of fungicides.

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**Fig 1.** Colony morphology and lesion on tomato fruits produced by *B. cinerea* isolates

**Fig 2.** EC50 (µg/ml) of *B.cinerea* isolates resistance and sensitive to boscalid (BC-1101, BC-1102)R, (BC-1105)S , (BC-1103, BC-1104)MR.

**Fig 3.** EC50 (µg/ml) of *B.cinerea* isolates resistance and sensitive to fluopyram (BC-1101, BC-1103)R, (BC-1105)S , (BC-1102, BC-1104)MR.

**Fig 4.** EC50 (µg/ml) of *B.cinerea* isolates resistance and sensitive to carbendazim (BC-1103)R, (BC-1105)S , (BC-1101,BC-1102, BC-1104)MR.

**Fig 5a**. Amplification of *Sdh*-B gene

**Fig 5b**. Amplification of β-tubulingene

**Fig 6**. *In vitro* pathogenicity of *B.cinerea* isolates on tomato fruits. Different letters indicates the significant different between each isolates according to L.S.D at P=0.05

**Fig 7**. *In vitro* sporulation of different isolates of *B.cinerea*. Values followed by different letters indicate the level of significant difference between isolates according LSD at P=0.05

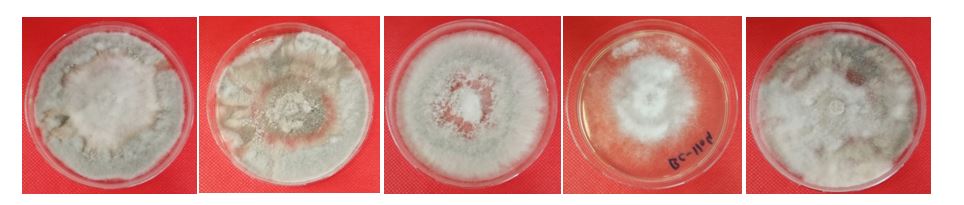
**Fig 8a.** Spore germination of *B. cinerea* isolates. Values followed by similar letter indicate no significant difference between each isolates according to LSD test at P=0.05

**Fig 8b.** Conidia germination of *B. cinerea* isolates

**Fig 9.** Sensitivity of *B. cinerea* isolates mycelial growth at different temperature

**Fig 10.** Mean lesion diameter of the pathogenicity of *B.cinerea* pathogenicity in greenhouse

**Table 1.** Fungicides used in this study with chemical class and mode of action



BC-1105

BC-1103

BC-1104

BC-1101

BC-1102



**Fig 1.** Colony morphology and lesion on tomato fruits produced by *B. cinerea* isolates

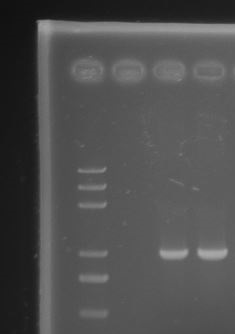
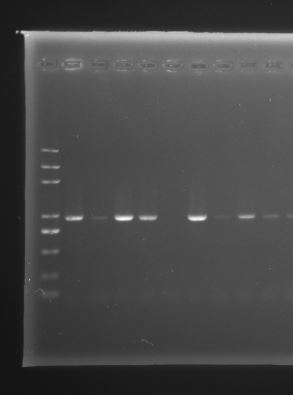
Fig 2. EC50 (µg/ml) of *B.cinerea* isolates resistance and sensitive to boscalid (BC-1101, BC-1102)R, (BC-1105)S , (BC-1103, BC-1104)MR.

*B. cinerea* isolates

EC50 μg/ml

Fig 3. EC50 (µg/ml) of *B.cinerea* isolates resistance and sensitive to fluopyram (BC-1101, BC-1103)R, (BC-1105)S , (BC-1102, BC-1104)MR.

Fig 4. EC50 (µg/ml) of *B.cinerea* isolates resistance and sensitive to carbendazim (BC-1103)R, (BC-1105)S , (BC-1101,BC-1102, BC-1104)MR.

M

S

R

S

R

M

950bp

1000bp

**Fig 5a**. Amplification of *Sdh*-B gene **Fig 5b**. Amplification of β-tubulingene

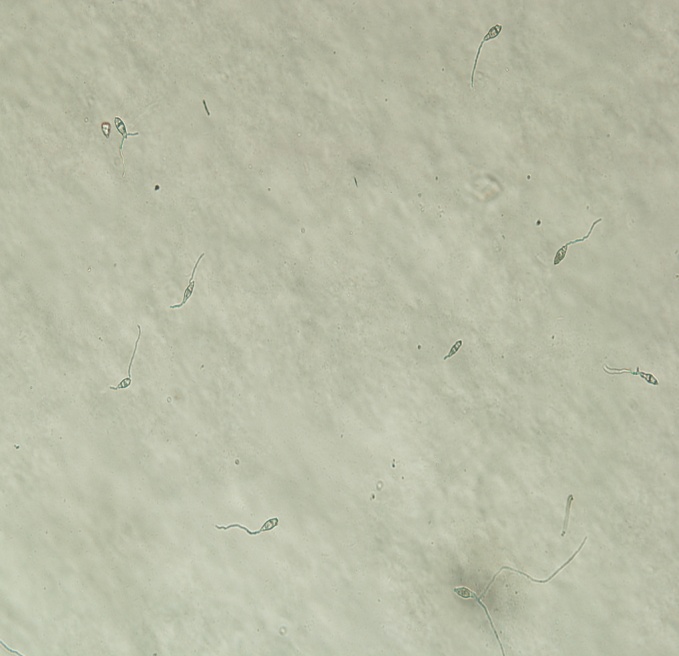
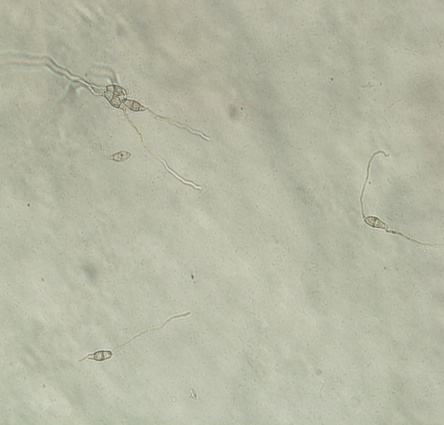
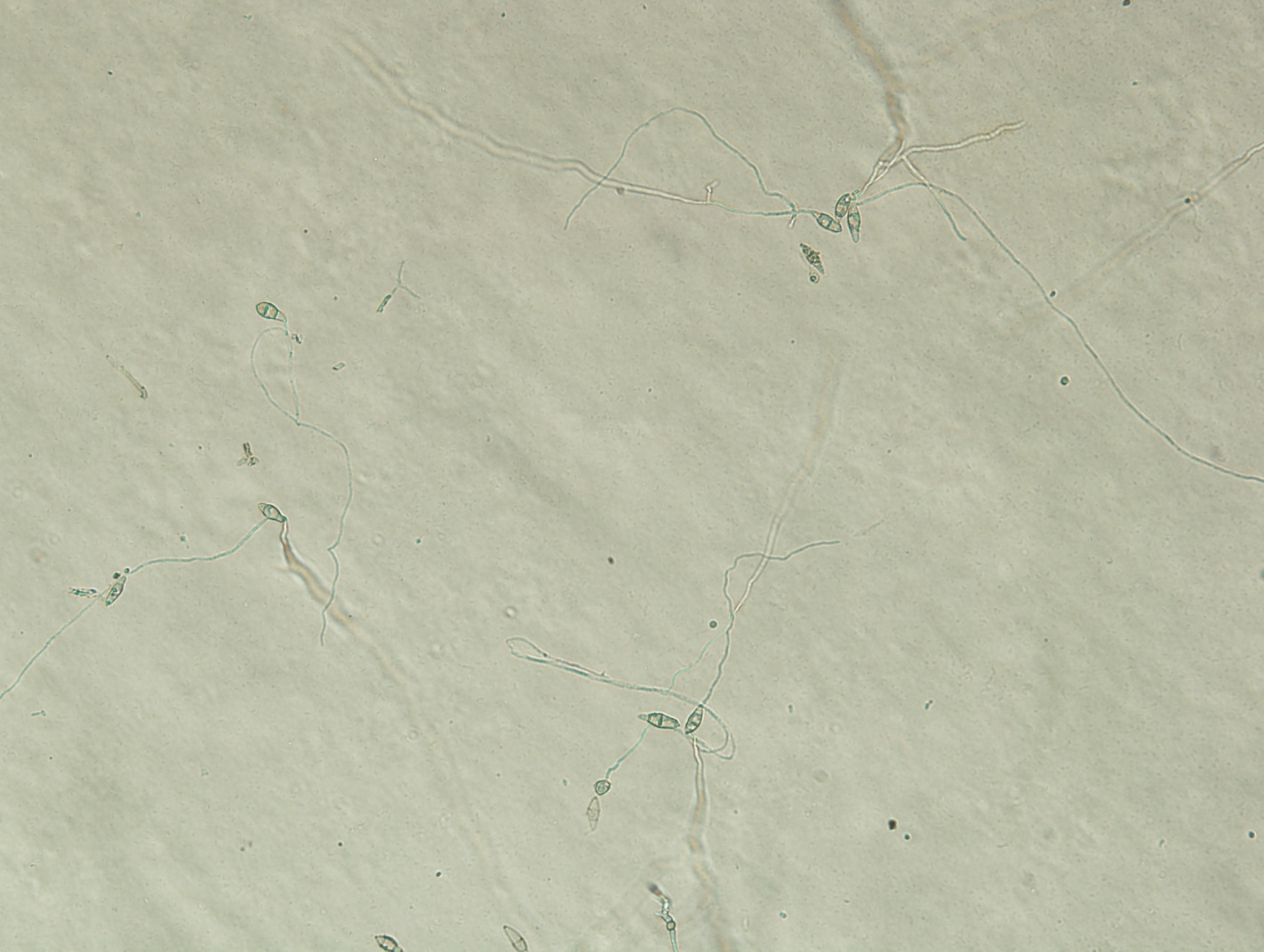
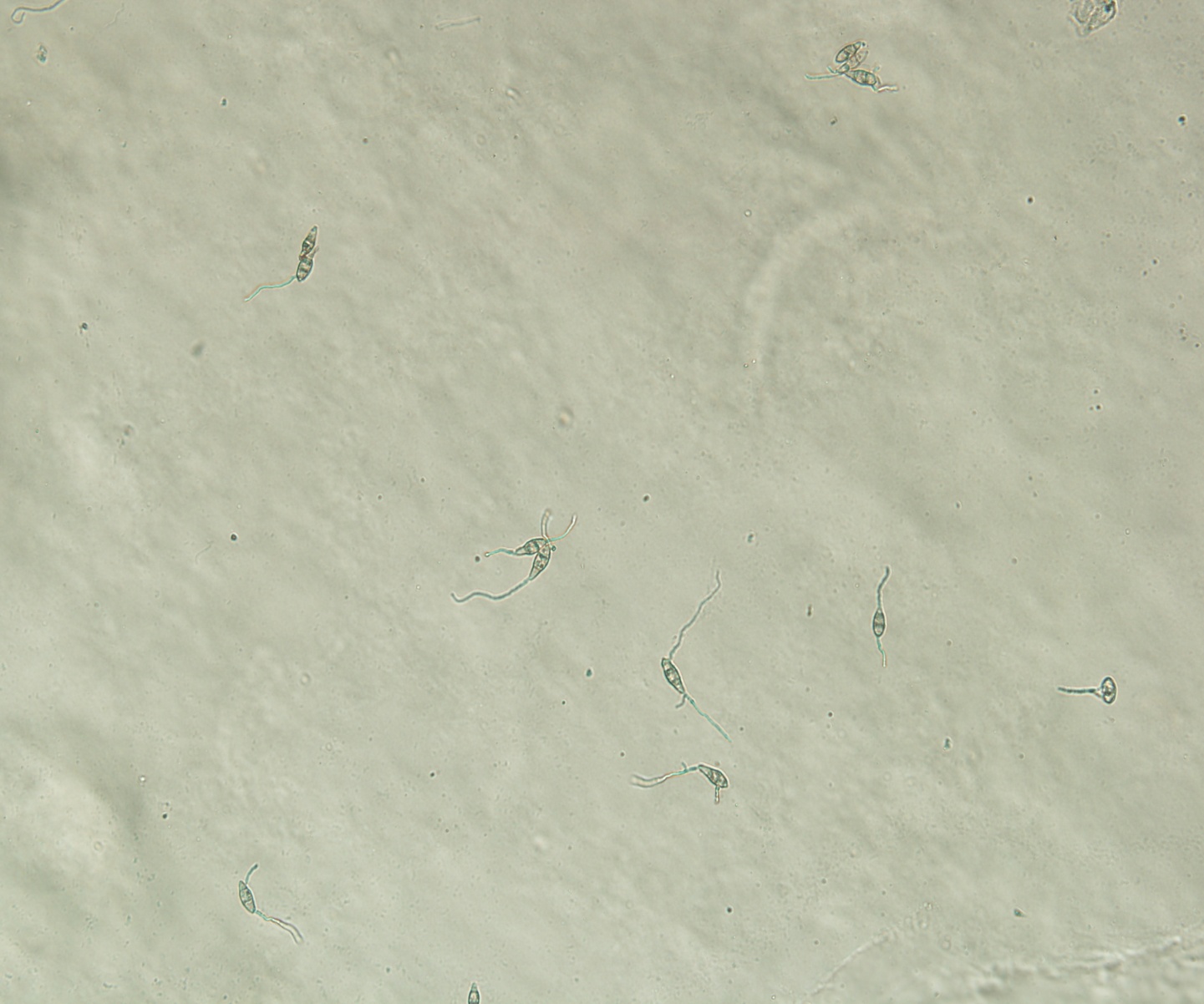
**Fig 6**. *In vitro* pathogenicity of *B.cinerea* isolates on tomato fruits. Different letters indicates the significant different between each isolates according to L.S.D at P=0.05

Isolates of *B. cinerea*

Sporulation (cm2)

**Fig 7**. *In vitro* sporulation of different isolates of *B.cinerea*. Values followed by different letters indicate the level of significant difference between isolates according LSD at P=0.05

Fig 8a. Spore germination of *B. cinerea* isolates. Values followed by similar letter indicate no significant difference between each isolates according to LSD test at P=0.05

**BC-1103**

**BC-1102**

**BC-1101**

**BC-1105**

**BC-1104**

Fig 8b. Conidia germination of *B. cinerea* isolates

Fig 9. Sensitivity of *B. cinerea* isolates mycelial growth at different temperature

Fig 10. Mean lesion diameter of the pathogenicity of *B.cinerea* pathogenicity in greenhouse

Table 1. Fungicides used in this study with chemical class and mode of action

|  |  |  |  |
| --- | --- | --- | --- |
| **Isolate** | **Bos** | **Fluo** | **Car** |
| BC-1101 | R | R | MR |
| BC-1102 | R | MR | MR |
| BC-1103 | MR | R | R |
| BC-1104 | MR | MR | MR |
| BC-1105 | S | S | S |