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Molecular Characterization, Etiology of *Sclerotinia sclerotiorum*, an Emerging Fungal Pathogen of White Mold Disease in Eggplant and its Biocontrol Using Soil Bioagents

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Sclerotinia sclerotiorum causes white mold disease, that result in losses of up to 50% in several host plants worldwide. It is a harmful fungus as it infects plants at any stage of development, in the field, or storage; this includes young seedlings, mature plants, and fruits. The accessibility of molecular and biological methods has contributed significantly to our understanding of *S. sclerotiorum* etiology and progression. The present research aimed to characterize *S. sclerotiorum* causing white mold disease in eggplant (*S. melobgena*), evaluate the potentiality of some bio-agents for inhibiting the growth of the fungus and develop a strategy for dealing with the disease challenge. Eggplants grown in open fields and greenhouses showed cottony growth on their stems, followed by the emergence of sclerotia. A fungal growth with fluffy mycelium and considerable sclerotia was isolated from the infected tissues. Polyphasic characterization, and phytopathological analysis identified the fungus as *S. sclerotiorum*. The symptoms previously seen in the field were reproduced by inoculating healthy eggplant roots, stems, and leaves with the fungus. The ITS rDNA sequencing (564 bp) homology and phylogenetic analysis showed 99% sequence similarity of the isolate with multiple *S. sclerotiorum*. A wide range of strategies are required for disease management. *S. sclerotiorum* is sensitive to competition from other bacteria. Such a technique might act as an initial basis for effective biological control of white mold. The results demonstrated that eight isolates have very strong antagonistic activity against *S. sclerotiorum*. Reduced rates ranged from 63.1% (65 D) to 39.0.1% (75 A1). At the same time, the effect of the *Trichoderma harzianum* fungus against the pathogen *S. sclerotium* was apparent in restricting mycelium growth and inhibiting the fungus growth. ITS sequencing identified the mycopathogen. The interaction of seven *Bacillus* spp isolates with *S. sclerotiorum* on PDA culture media highlighted these isolates' capacity to limit the pathogen's mycelial growth. Furthermore, the antimicrobial agent must remain in the same environment for an extended period while also being active against the pathogen.

Keywords: Biocontrol; Eggplant; ITS; Bio-agents; *Sclerotinia sclerotiorum*; Pathogenicity.

1. Introduction

Eggplant (*Solanu melongena* L.) is the main agricultural product that has gained remarkable popularity through the previous century. Over 90% of the world's eggplant is produced in five countries: China, India, Egypt, Iran, and Turkey (FAO, 2011). The local production of eggplant in Saudi Arabia is estimated at 4,100 ha/MT (Ministry of Environment Water and Agriculture, 2020). Eggplants are high in fiber and antioxidants. Eggplant provides a minimum of 5% of a person's daily needs of fiber, manganese, copper, B-6, and thiamine requirements (Noda *et al.*, 2000; Whitaker and Stommel, 2003). Plant diseases are estimated to cause damage to 10% of all eggplants worldwide each year. This may result in significant financial losses for farmers and society in

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developing countries (Strange and Scott, 2005). The production of eggplants is seriously threatened by *S. sclerotiorum* because there are few or no resistant types of eggplant. One of the most common and devastating white diseases caused by *Sclerotinia sclerotiorum*. Worldwide, the fungus affects more than 400 plant species, including important crops and plenty of weeds. Typical Sclerotinia lesions on leaves and leaf axils include water-soaked areas or lesions that appear light grey-white or brownish-white. Water-soaked sores may rapidly spread above and below the infected plants, along and around the stems. Sclerotinia-affected plants first develop a white, cottony growth along their stems, and then they start to produce sclerotia, which can grow inside or outside of the stem (Fernando *et al.*, 2004; Bolton *et al.*, 2006; Kamal *el al.*, , 2016; Willbur *el al.*, 2019). *S. sclerotiorum* can attack plants at all stages of development. Sclerotium infections often show scattered patches throughout a field, and symptoms are not visible until late in the growing season, after the flowering stage (Derbyshire and Denton-Giles, 2016; Grau *el al.*, 2004). *S. sclerotiorum* can infect various plant species and tissues in various environmental conditions. It can also produce sclerotia, which may remain in the soil for several years and contribute to the pathogen's persistence and spread (Bolton *el al.*, 2005; Bardin Huang, 2001). Numerous studies conducted in the nation have previously reported the morphological characteristics of *S. sclerotiorum* isolates obtained from several hosts (Rahman *el al.*, 2020). The genetic diversity of the fungus was determined using a variety of molecular techniques, including sequence-related amplified polymorphism (SRAP) technique (Li *el al.*, 2009), random amplified fragment length polymorphism (Thilagavathi *el al.*, 2013), microsatellite marker (Meinhardt *el al.*, 2002), amplified fragment length polymorphism (Cubeta *el al.*, 1997), Universal Rice Primer Polymerase Chain Reaction (URP-PCR) (Aggarwal *el al.*, 2008), and sequence divergence within the ribosomal DNA (rDNA) internal transcribed spacer (ITS) (Carbone and Kohn, 1993). The disease can be managed through cultural, biological, and chemical methods and the use of resistant varieties. Several biocontrol agents (BCAs) have been described as effective against *S. sclerotiorum* (Hjeljord and Tronsmo, 1998).—*Pseudomonas* sp. inhibit the germination of ascospores of *S. sclerotiorum* by antibiosis (Fernando et al., 2007). The suppression of *S. sclerotiorum* by *Trichoderma harzianum* (Th38) and *Epicoccum purpurescence* was due to colonization of the petals of brinjal (Singh and Kaur, 2001). In the white mold of pea induced by *S. sclerotiorum*, *T. harzianum* has been reported to parasitize on the mycelium and sclerotia of *S. sclerotiorum* and destroy the sclerotia within 15 days (Sumida *el al.*, 2015). A potential biological control agent (BCA) against *S. sclerotiorum* was assessed *in-vitro* and *in-vivo* using an isolate of *T. harzianum* and two isolates of *Bacillus amyloliquefaciens*. The findings indicated that the growth and production of mycelia and sclerotia were inhibited by *T. harzianum* and *B. amyloliquefaciens* (Abdullah *el al.*, 2008).

Current study aimed to characterize the *S. sclerotiorum* causing white mold disease in eggplant (*S. melobgena* L.) molecularly and biologically, as well as to evaluate the potentiality of some bio-agents for inhibiting the fungus' growth and develop a strategy to deal with the disease challenge.

2. Materials and Methods

2.1. Samples collection and pathogen isolation

Eggplant samples showing symptoms of sclerotinia white mold were collected from eggplants cultivated under greenhouses in Al-Qassim region. Ten infected plants were collected from each greenhouse, put in plastic bags, and transferred to laboratory The tissues of the infected stem, roots, and fruits were removed, and after being cut into 2-3 mm pieces, they were surface sterilized with 70% ethanol for 30 seconds, rinsed three times in sterile distilled water, and dried on blotter paper. The tissue fragments were inserted in petri plates containing potato dextrose agar (PDA). Petri dishes were kept in the dark at 25ºC for 5 days. *S. sclerotiorum* colonies were purified for future investigation.

2.2 Phenotypic characterization of *S. sclerotiorum* isolates

Morphological characteristics of fungal purified isolates, including mycelial structure, sclerotial features, conidia, conidiophore, peculiar forms of apothecia, and ascospores, were evaluated for pathogen identification at both asexual and sexual stages. A single hyphal end of a fungal isolate was grown on fresh PDA dishes for a week. The fungus colony features and sclerotial shapes were observed with the naked eye. Surface-sterilized sclerotia were cultivated in 9 cm diameter Petri dishes filled with sterile, humid sand for 5-6 weeks at 4 ^{o}C, then the morphological characteristics of apothecia and ascospores formed by sclerotia were examined under the microscope (40X and 100X).

2.3. Pathogenicity tests

2.3.1. Pathogenicity test on vegetative plant

Three *S. sclerotiorum* isolates, namely QB1-QB2-QB3, were chosen for pathogenicity. The eggplant seeds were thoroughly sterilized before being placed in the growth chamber in circular plastic pots filled with sterile soil. PDA plugs cut from actively growing edges of fungal colonies were placed in wounds formed in the stem bark of 4-week-old-eggplant seedlings about 10 cm above the soil level with a sterile blade and sealed with moistened cheesecloth. Four week old eggplant seedlings were infected with sterile (PDA) plugs as control. The inoculated seedlingswere then covered with plastic bags and kept in a dark growth environment at 23-25°C for 48 hours. Twenty plants were inoculated with *S. sclerotiorum* isolates, while five sereved as controls. The fungus was re-isolated from symptomatic tissues to confirm that the original and re-isolated fungus matched the same Koch's hypothesis.

2.3.2. Pathogenicity test on root

Six-week-old eggplant seedling with three true leaves were transplanted singly into 16 cm diameter plastic pots containing soil and treatment. The pots then were inoculated with QB1-QB2-QB3 *S. sclerotiorum* isolates QB1, QB2 and QB3 which were grown for 7–10 days on PDA. Controls pots were inoculated with sterile (PDA) plugs. All pots were kept in an agricultural room at 23–25 °C until disease synmptoms appearance. The pathogen was re-isolated from the roots and stems of infected plants to verify Koch's hypothesis.

2.3.4 Pathogenicity test on detached eggplant leaves

Small pieces of sterile cotton were placed inside Petri dishes, then sterile filter paper was placed over the cotton and wetted with sterile distilled water. Surface sterilized eggplant leaves were then placed in Petri dishes. Twenty eggplant leaves (without any scratches) were inoculated with 10 mm diameter cultured agar discs of QB3 *S. sclerotiorum* and incubated at 25^{o}C for three days in a moist petri dish, followed by recording the observations.

2.4. Molecular identification of *S. sclerotiorum*

2.4.1. DNA extraction

Cetyltrimethylammonium bromide (CTAB) extraction protocol described previously by (Maixner *et al.,* 1995) was used for DNA extraction from purified *S. sclerotiorum* isolates. The mycelium of 14 isolates of *S. sclerotiorum* (Table 1) was harvested from the surface of 7-day-old potato dextrose agar plate cultures. One hundred milligrams of mycelial mate were grounded in liquid nitrogen and used to extract total genomic DNA. The pure DNA was eluted in a final volume of 50 µl. The NanoDrop ND-1000 spectrophotometer was used to evaluate the concentration and purity of isolated DNAs. The DNA was stored at -20ºC.

2.4.2. Condition and amplification of PCR, DNA sequencing, and Phylogenetic analysis

Using ITS4/ITS5 pair primers (White *et al.,* 1990), internal transcribed spacer (ITS) region amplification of 14 *S. sclerotiorum* isolates rDNA was carried out in an automated thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems) in the Plant Pathology Lab., Faculty of Agriculture and Veterinary, Qassim University. The PCR program consisted of 35 cycles of 94 °C for 30 s, 51 °C for 1 min, 72 °C for 1.5 min, and a final extension at 72 °C for 3 min. The 40 µl PCR mixture contained the following: 1 µl of 40 ng nucleic acid, 20 µl of GoTag®Colorless Master Mix (Promega Corporation, USA), one µl of each primer (10 pmol), and 17 µl of nuclease-free water (Promega). The amplified PCR products were electrophoresed for analysis using a 1.5% agarose gel containing 3 µl of ethidium bromide (10 mg/ml, Bio-Rad), and a UV transilluminator was used to visualize the DNA bands. (G: BOXF3 system, Syngene). The PCR products were sent to Macrogen Inc. (Korea) for direct sequencing in both directions with ITS4/ITS5 primers (White et al. 1990). Raw sequences were assembled and modified using GAP4 (Bonfield et al. 1995). ClustalW was used for multiple alignments of sequences (Thompson *et al.,* 1994), and the Neighbor-Joining method, using MEGA6 software, was used to draw the phylogenetic tree (Tamura *et al.,* 2007). The ITS4/ITS5 sequences of different *Sclerotiorum* species from several countries used for comparisons were retrieved from GenBank (www.ncbi.nlm.nih.gov).

2.5. Isolation and identification of local rhizosphere bio-agents

Bacterial and fungal bioagents were isolated from healthy plant soil rhizospheres in AI-Qassim regions, including Buridah, Unayzah, Addalfaah, al Bukayriyah, Alrass, Albadai, and Alshamasiyah, Saudi Arabia, by serial-dilution method on nutrient agar (NA) and PDA media. One hundred soil samples were collected. After drying and sieving, 2-5 g of each soil sample was used to prepare a suspension in a tube in 9 ml of sterile physiological water. Once the homogenization is done, a series of dilutions were prepared in sterile physiological water, and 100 µl of each dilution was separately plated on the NA medium. The Petri dishes were incubated at 28–30 °C and observed for 1–3 days.

After incubation, bacterial isolates were distinguished from other microbial colonies by characteristics such as tough colonies partially submerged in the agar (Wang *et al.*, 2016). The 16S rRNA gene was employed to identify bacterial bioagent isolates. In accordance with (Cook and Meyers, 2016) method, DNA will be extracted from cultures grown in NB for 24 hours. The 16S rRNA region of bacterial isolates was amplified using the universal pair primers 27F/1492R. Fungal isolates were distinguished and transferred to PDA media, purified, and identified, as previously explained with *Sclerotiorum* isolates.

2.6. Assessment of the select bacterial bio-agents on *S. sclerotiorum* isolates *in vitro*

Evaluation of several bacterial bio-agents on mycelial growth and sclerotia production of *S. sclerotiorum*. The antagonistic capabilities of some bioagents were tested against the pathogenic fungus isolate, *S. sclerotiorum*, by adding 1 ml bacterial suspension of 3 days old following culture of each baccterial bioagent isolate, n the center of Petri dish cotaning PDA medium and moving it circularly to distribute the suspension homogeneously before adding a disc of 0.5 cm in diamter from the pathogenic fungus culture, with following the completion of fungus growth in the control treatment, The pathogenic fungus's growth rate and the percentage of inhibition were estimated using the formula below:

Where,

r1 is the radial growth of the control,

r2 is the radial growth of the co-culture

Preparing effective microorganisms (EM-1) and testing its antagonistic activity against the *S. sclerotiorum* fungus was carried out according to Yan *et al.* (2015).

2.7. Evaluation of the effectiveness of treatments involving *Trichoderma harzianum*

The antagonistic effect of the *Trichoderma* fungus on the *S. sclerotiorum* fungus was tested by placing discs of *Trichoderma* fungal growth on both sides of the Petri dish, then placing a disc of *S. sclerotiorum* fungus between them in the middle of the Petri dish. Plates were incubated at 18 ± 2 °C and lighting for 16 hours daily. The treatments were evaluated by measuring diameter of radial growth of the pathogenic *S. sclerotiorum* . Then, mean diameter of radial growth pathogen diameter was calculated and photographed.

2.8. Statistical aalyses

The XL ST (Version 2012) and Microsoft Office Excel 200software packages were utilized for statistical analysis. All studies in this study were designed to be completely randomized, with three replicates for each treatment. The experiment was repeated at least twice, and means of the treatments were separated using a t-test or Fisher's LSD test (0.05).

3. Results

3.1. Symptoms of white mold on eggplant plants

Approximate 5% of the eggplants in each surveyed field exhibted disease signs and symptoms. The infected plants had matured physiologically, but they had not yet died. Internal and external large brown patches with necrotic tissues were visible on infected stems and fruits (Fig. 1 a & d).

Some diseased plant died early and bleached (Fig. 1b). On infected tissues, fluffy white mycelia were observed, and black sclerotia formed on the surface of diseased tissues (Fig. 1c). The sclerotia were variable in size ranging in length from 2.9 to 15.2 mm and width from 2.2 to 5.2 mm.

3.2. Isolation and Morphological Characterization of Sclerotinia

S. sclerotiorum colonies growing PDA were rapidly expanding, coloring of white, floccose, aerial mycelia, with reversal reddish buff (Fig. 2b). The hyphae were hyaline, branching, and multinucleate under the microscope (Fig. 2d). There were no conidia or conidiophores formed. Sclerotia formed concentric rings and radiating lines at the colonies' expanding borders in culture. They ranged in shape from globose to cylindrical with a black outer rind and a white inner cortex. Each PDA Petri plate contained around 30 to 40 sclerotia (Fig. 2a). Throughout the process of sclerotial development, three macroscopically different stages were detected. Sclerotia were white at the start (initiation), beige after a few days of growth (development), and black at maturity (maturation) (Fig. 2a & c). Apothecia appeared after 3 to 5 weeks of incubation. Single to several apothecia developed from a sclerotium, and their colors ranged from brown to amber. When juvenile, receptacles were large, somewhat concave when grown, and convex with a central depression (Fig. 2b). Apothecian asci were cylindrical and eight-spored (Fig. 2b). Uniseriate ascospores were single-celled, hyaline, and ellipsoid (Fig. 2d).

Fig. 1. *Sclerotinia sclerotiorum* Field infected eggplant. (a) whitish mycelium growth and sclerotial formation on the infected stem (b) Sclerotinia causing drying of the stem; (c) Infection of white mold on eggplant fruit; (d) White mold infection on basal stem portion.

3.3. Pathogenicity test on vegetative plant

Three weeks after the inoculation, water-soaked lesions around the inoculation site developed on the inoculated plants, and the lesions spead throughout the stem, forming a noticeable distinguishing border between healthy and infected tissue (Fig. 3a). The lesions were present on all plants after four weeks; moreover, all plants had wilted and collapsed, with some dead plants showing stem breaking (Fig. 2b). On and inside the infected stems, sclerotia of different shapes and size were formed which are typical of *S. sclerotiorum*. Dead plant fragments formed white mycelia and sclerotia in a damp chamber. Control plants showed no signs of diseases (Fig. 3a &b). Pathogenicity testing on eggplants demonstrated that symptoms similar to those noticed in the farmer's field occurred 30 days after inoculation. The fungus was re-isolated from diseased plant tissues, and the colonies and fungal morphologies matched those found in field-infected plants. (Fig. 3c).

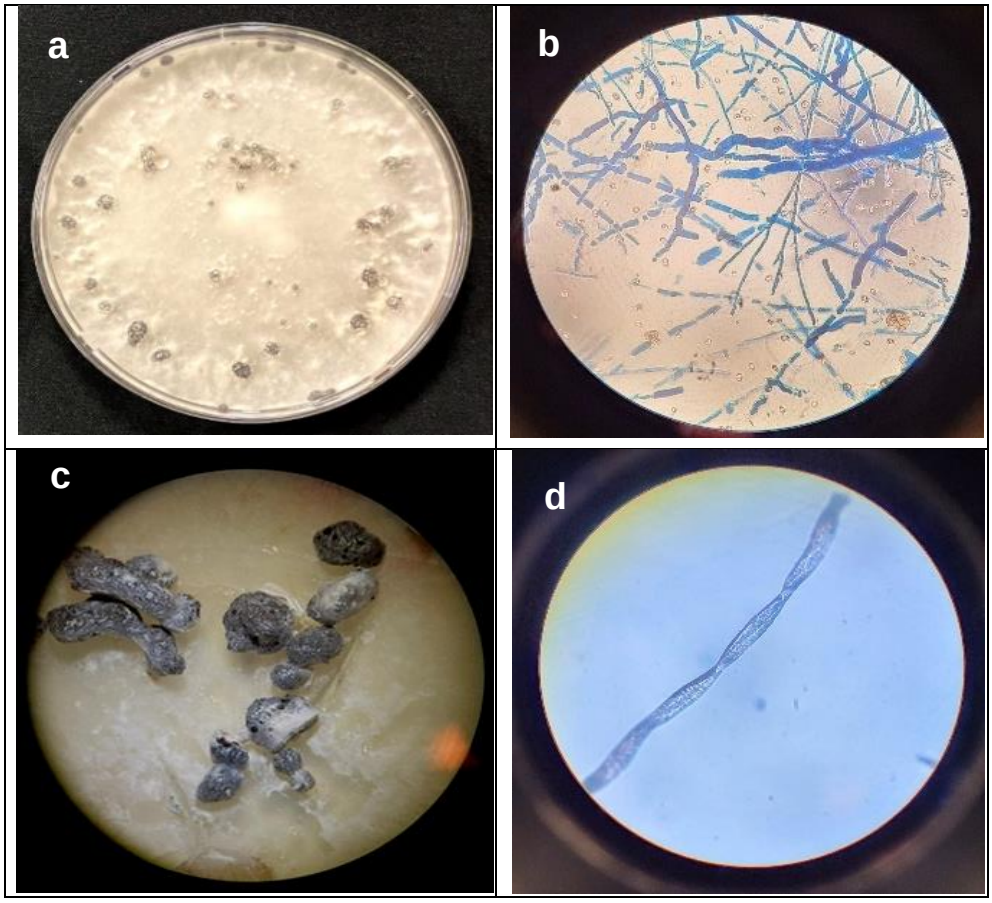

Fig. 2. (a) Pure culture of the infected *S. sclerotiorum* showing white fluffy mycelium and sclerotial bodies, (b) conidiophore with conidia (c) forms of sclerotia. (d) *S. sclerotiorum* hyaline mycelium micrograph.

Fig. 3. (a) Inculated eggplants with *S. sclerotiorum* showing water-soaked lesions near the inoculation site, (b) all plants had wilted and collapsed, with some dead plants showing stem breaking, and (c) colony and fungal morphologies of re-isolated from symptomatic plant tissues.

3.4. Pathogenicity test on roots

Typical stem rot symptoms were observed on the inoculated plants. Direct application of the mycelial plug inoculum near the plant's root and crown stem resulted in a higher percentage of infection (75-81%). On the other hand, using sclerotia as inoculum was found to be less infective.

Disease symptoms appeared 5 to 10 days after inoculation as small, grayish, water-soaked lesions, which quickly enlarged along the stem and developed into patches of soft rotting tissues. The affected stems were covered with whitish mycelial mats and black sclerotia were observed in the pith in various sizes when the diseased stem was split.

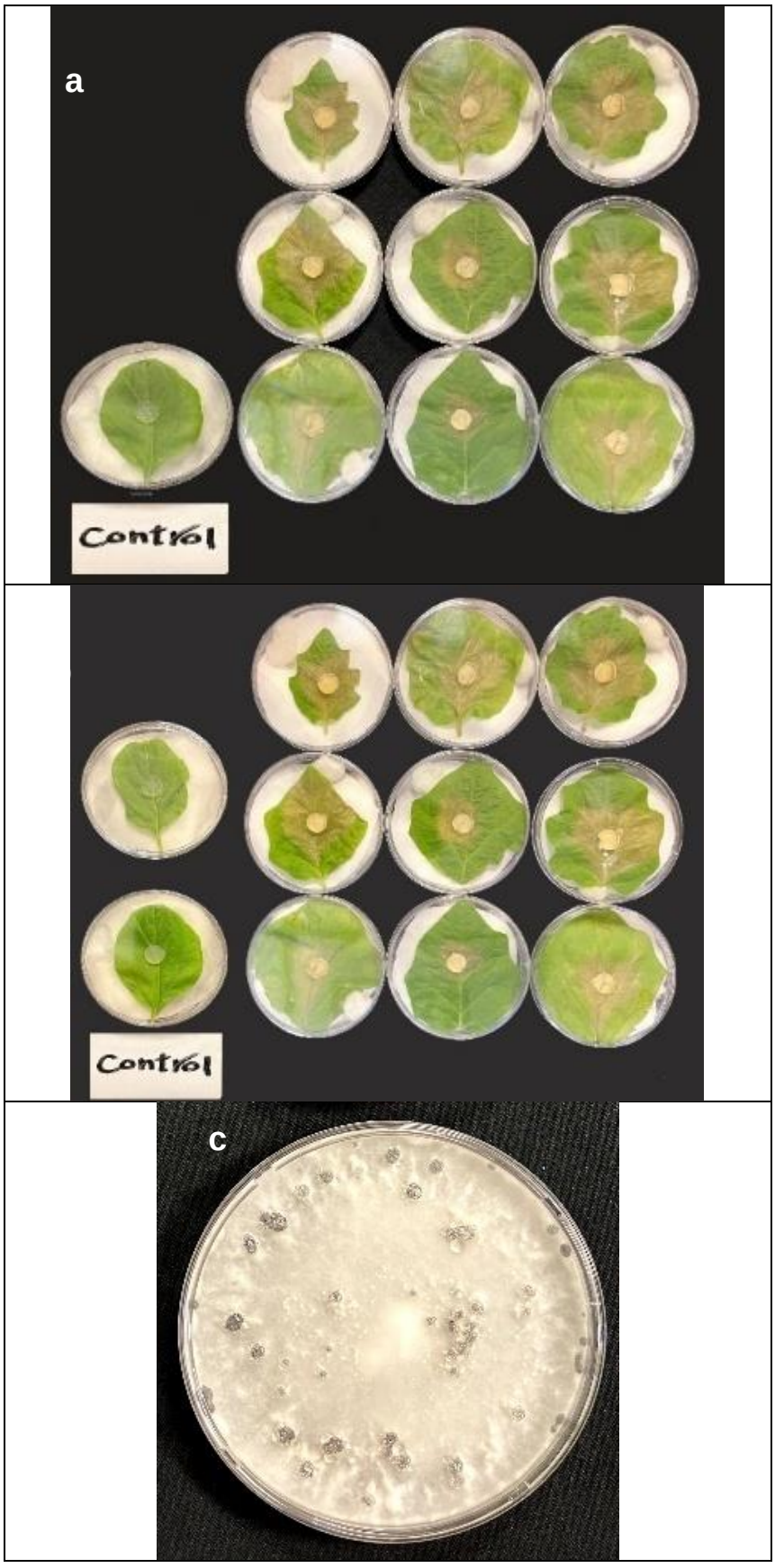

Fig. 4. (a) Pathogenicity test of eggplants detached leaves inoculated with PDA discs colonized with *S. sclerotiorum* showing a water-soaked lesions near the site of inoculation, (c); culture of re-isolated fungal pathogen from infected plant tissues.

3.5. Pathogenicity test on detached eggplants leaves

Nine eggplant leaves of 30 days-old eggplants were inoculated with 10 mm of surface culture agar discs of the QB3 isolate of *S. sclerotiorum*. Seven days after incubation in a moist petri dish at 25-23 °C, photographs were taken. Detached eggplant leaves inoculated with *S. sclerotiorum* turned brown on the third day, and lesions triggered to grow and develop downward across the veins on the fourth day. lesions were similarly larger on the ninth day (Figure 4a, b). On the other hand, the control remained healthy and green, with no signs of diseaese (Figure 4c).

3.6. Molecular identification of *S. sclerotiorum* isolates.

The fungal-specific primers pair ITS4-ITS5 was utilized to amplify the internal transcribed spacer (ITS) region of 14 *S. sclerotiorum* Saudi arabia isolates collected from infected eggplants and a size of approximately 564 bp was obtained (data not shown). The direct sequencing of both DNA strands of all PCR product samples of all *S. sclerotiorum* isolates were sequenced using ITS4 and ITS5 primers. The resulting sequences were deposited in GenBank with accession numbers shown in Table 1. The analysis of rDNA data using the NCBI BLAST tool highlighted morphological variability and affirmed the individual identities of the isolates. Furthermore, searches in the NCBI GenBank database revealed a 99–100% closest match with *S. sclerotiorum*. The nucleotide sequences resulting from the DNA products confirmed that all the fungal isolates were *S. sclerotiorum* using the BLAST algorithm. Searching for homology in the GenBank DNA database indicated that the eggplant Saudi Arabia *S. sclerotiorum* isolates ITS rDNA sequence shared 99% sequence identity with other worldwide *S. sclerotiorum* isolates. All *S. sclerotiorum* isolates employed in this investigation generated a singular amplicon without any length discrepancies. Notably, there were no observable differences among the isolates examined in this research, likely due to their shared origin from the same host species (Eggplant) and location (Qassim) within the same year.

Table 1. The isolates of *S. sclerotiorum*. used to screen for specificity of ITS4/ITS5 primers.

Isolate name	GenBank accession	Organism	Host	Gegrophical region
Qb1	OR527162	*S. sclerotiorum*	*S. melongena* L	Qassim, Buraydah
Qb2	OR527163	*S. sclerotiorum*	*S. melongena* L	Qassim, Buraydah
Qb3	OR527164	*S. sclerotiorum*	*S. melongena* L	Qassim, Buraydah
QBu1	OR527165	*S. sclerotiorum*	*S. melongena* L	Qassim, Bukiryah
QBu2	OR527166	*S. sclerotiorum*	*S. melongena* L	Qassim, Bukiryah
QBu3	OR527167	*S. sclerotiorum*	*S. melongena* L	Qassim, Bukiryah
QBu4	OR527168	*S. sclerotiorum*	*S. melongena* L	Qassim, Bukiryah
Qd1	OR527169	*S. sclerotiorum*	*S. melongena* L	Qassim, Dalfaah
Qd2	OR527170	*S. sclerotiorum*	*S. melongena* L	Qassim, Dalfaah
Qd3	OR527171	*S. sclerotiorum*	*S. melongena* L	Qassim, Dalfaah
Qd4	OR527172	*S. sclerotiorum*	*S. melongena* L	Qassim, Dalfaah
Qd5	OR527173	*S. sclerotiorum*	*S. melongena* L	Qassim, Dalfaah
QS1	OR527174	*S. sclerotiorum*	*S. melongena* L	Qassim, Alshehih
QS2	OR527175	*S. sclerotiorum*	*S. melongena* L	Qassim, Alshehih

3.7. Phylogenetic analysis

Clustal-X was used to align the acquired sequences using MEGA (version 6.0 software), and a neighbor joining tree was produced using the same program. The ITS region sequence of all *S. sclerotiorum* isolates was compared togaether and with other ITS sequences from different worldwide Sclerotinia species reported in GenBank (www.ncbi.nlm.nih.gov). The phylogenetic results (Figure 6) revealed that all 14 *S. sclerotiorum* isolates have been grouped together in one group with outstanding bootstrap support.

The phylogentic tree generated with other 19 isolates of *S. sclerotiorum* from diferent country and *Pseudoperonospora cubensis* serving as an out group (FJ304668). The phylogenetic tree revealed all the Saudi Arabia *S. sclerotiorum* isolates formed clade I with other 15 *S. sclerotiorum* (Fig. 6), however the two isolates of *S. rolfsii* separated in clade II and the *S. cepivorum* joined together in clade III (Fig. 5). *Pseudoperonospora cubensis* isolate stood alone out of *Sclerotinia* isolates. The majority of *S. sclerotiorum* isolates identified in the present study differ genetically from one another in certain nucleotide sequence areas and from other *S. sclerotiorum* isolates registered at NCBI (Figs. 6 and 7).

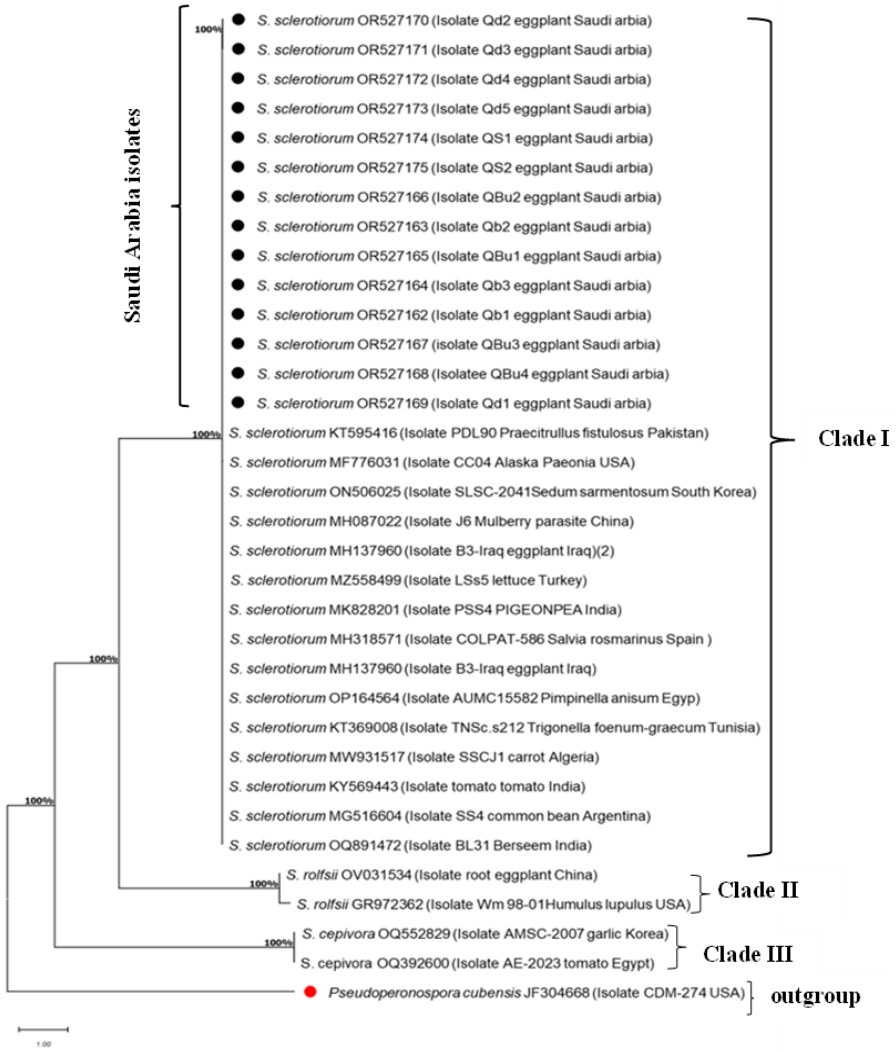

Fig. 5. Phylogenetic tree based on ITS region sequences constructed by the neighbor-joining method from 14 *S. sclerotiorum* isolates from Qassim province (marked in black color) and other 19 *Sclerotinia* species belonging to different geographical region available in the GenBank database. *Pseudoperonospora cubensis* (FJ304668) was used as the outgroup to root the tree. Bootstrap analyses were done in 500 replicates. Phylogenetic analyses were performed using the MEGA6 software.

Additionally, it was coming out that isolates QB1(OR527162), QB2(OR527163), QBu4 (OR527168) and QB3(OR527164) had a 99% similarity rate, since the isolates OR527165, OR527168, OR527171, OR527172 and OR527175 are genetically similar. However, nucleotide sequence analysis of *S. sclerotiorum* isolates OR527163 and OR527164 indicated a similarity rate exceeding 99%, the other isolates OR527165, OR527166, OR527167, OR527168, OR527169, OR527170, OR527171, OR527172 and OR527173 showed a similarity percentage of up to 90% among them. The least genetic similarity (83%) was observed with sugarbeet isolate (MW375589) identified from USA. The isolates (QS2, Qd4, and QBu1) exhibited a 100% similarity rate with isolates identified to infect cabbage heads in India (Mz379262), while they showed the least similarity with other isolates recorded in the NCBI database, such as those isolated from green bea in South korea (MG931017), (MT177216) from Argentina and the Pakistan (MH457168) (Figures 7). Results also indicated that the remaining *S. sclerotiorum* isolates (QBu3, QS1, and QBu2) were genetically distinct from the other *S. sclerotiorum* isolates. Conversely, isolates (Qd5, Qd1, and Qd2) were closer genetically and showed the least similarity to *S. sclerotiorum* isolated from pigeon pea in India (MK8282201).

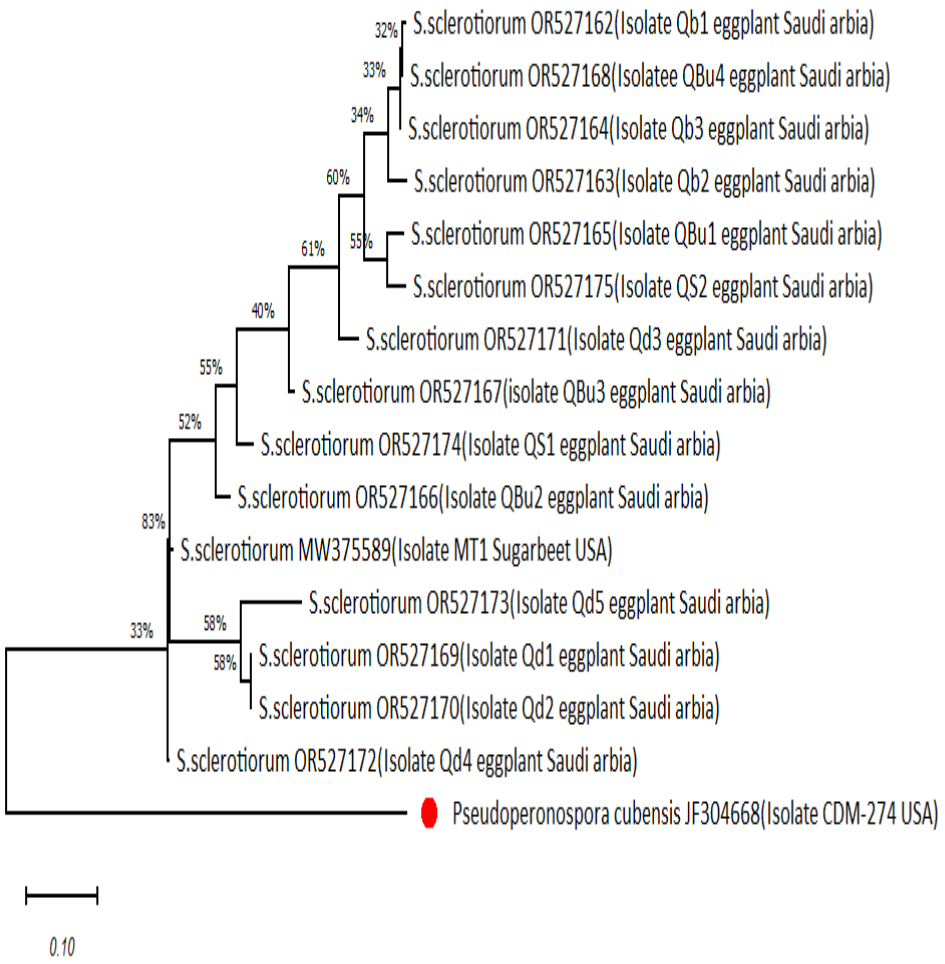

Fig. 6. Phylogenetic tree (Neighbor-Joining tree) shows the genetic relationship between the *S. sclerotiorum* isolate (1), identified in this study, and the other isolates registered at NCBI.

3.8. Isolation and identification of local rhizosphere bio-agents

102 Bacterial samples have been collected from the soil rhizosphere of healthy plants from different areas of Al-Qassim. After drying and sieving, 2 g of soil were used to prepare a suspension in 9 ml of sterile water. After homogenization, a series of dilutions in sterile water at a concentration of 1ml were produced and 50 l of each dilution was separately plated on NA media. After incubating Petri plates kept at 28-30°C for a period from 1 to 3 days, bacterial isolates were differentiated from other microbial colonies by properties such as tough colonies partially immersed in the agar. The outcomes demonstrated that *Bacillus* species represent, their counting revealed that they make up only a small percentage of the overall flora in the soil samples (a total of 102 isolates were chosen). The majority of these isolated *Bacillus spp*. isolates (79%) have white colonies. *Bacillu*s spp. that is brown, red, or gray is rare (1%), while 40 isolates (38.46%) colour the medium.

Table 2. The isolates of *Bacillus sp*. used to screen for antagonism against *S. sclerotiorum in-vitro.*

Isolate name	GenBank accession	Organism	Host	Gegrophical region
88 D	OR6448730	*B. subtilis*	*Washingtonia filifera*	Qassim, Buraydah
65 D	OR6448731	*B. subtilis*	*Phoenix dactylifera*	Qassim, Almethnb
75 A	OR6448732	*B. subtilis*	*Phoenix dactylifera*	Qassim, Onaizah
38 C1	OR6448733	*B.velezensis*	*Phoenix dactylifera*	Qassim, Buraydah
64 C2	OR6448734	*B. subtilis*	*Bromopsis pubescens*	Qassim, Almleda
86 C1	OR6448735	*Bacillus* sp	*Vachellia tortilis*	Qassim, Alqareen
60 B	OR6448736	*B.moiavensis*	*Calligonum comosum*	Qassim, Alrubiayah
65 C	OR6448737	*B. subtilis*	*Phoenix dactylifera*	Qassim, Almethnb
88 D	OR6448730	*B. subtilis*	*Washingtonia filifera*	Qassim, Buraydah

3.9. *Bacillus* spp. antagonism against *S. sclerotiorum In-vitro*.

Following sample screening, *Bacillus spp*. isolates (75 A, 60 B, 38 C1, 88D, 65C, 64C2, and 65D) against the fungus *S. sclerotiorum* were identified and submitted to the GenBank under the accession no; (OR648730, OR648731, OR648732, OR648733, OR648734, OR648735, OR648736, OR648737,). Each bacterial isolate was incubated for 24 hours at 282°C by being streaked as a band on the border of a PDA plate with a 90-mm diameter. The center of previously injected PDA plates was next inoculated with a 10 mm diameter mycelial disc of *S. sclerotiorum*, which was taken from the edge of a colony that was expanding. The Petri dishes were wrapped with parafilm and incubated at 28°C in darkness and at ambient temperatures for 6 days. As a control, plates with only the fungus's mycelial plug were kept.

According to the data presented in Table (3), two isolates (64 C2 & 86 C1) have a significant antagonistic effect on *S. sclerotiorum*. 51.8% and 56.2% respectively. The interaction of many isolates of *Bacillus* spp. with *S. sclerotiorum* on the PDA culture medium highlighted these isolates' capacity to prevent the pathogen.

Table 3. Summary of all pairwise comparisons for *Bacillus* Strains (Duncan).

***Bacillus* isolates**	**LS means (Fungal hypha (mm)**	**Groups**		
75 A	17.250	A		
60 B	16.125	A	B	
38 C1	15.875	A	B	
88 D	15.750	A	B	
65 C	15.625	A	B	C
64 C2	15.000		B	C
86 C1	14.250		B	C
65 D	13.875			C

***Bacillus* isolates**	**LS means (Distance between fungus to bacteria (mm)**	**Groups**		
65 D	27.813	A		
38 C1	25.250		B	
64 C2	23.875		B	
88 D	23.813		B	
75 A	23.750		B	
60 B	23.625		B	
86 C1	23.125		B	
65 C	22.938		B	

***Bacillus* isolates**	**LS means, fungus inhibition (%)**	**Groups**		
65 D	63.123	A		
86 C1	56.168	A	B	
64 C2	51.783	A	B	C
38 C1	50.230		B	C
88 D	48.148		B	C
65 C	46.640		B	C
60 B	45.268		B	C
75 A	39.038			C

***Bacillus* isolates**	**Fungal hypha (mm)**	**Distance between fungus to bacteria (mm)**	**fungus inhibition (%)**
38 C1	15.875	25.250	50.230
65 D	13.875	27.813	63.123
64 C2	15.000	23.875	51.783
88 D	15.750	23.813	48.148
75 A	17.250	23.750	39.038
60 B	16.125	23.625	45.268
86 C1	14.250	23.125	56.168
65 C	15.625	22.938	46.640

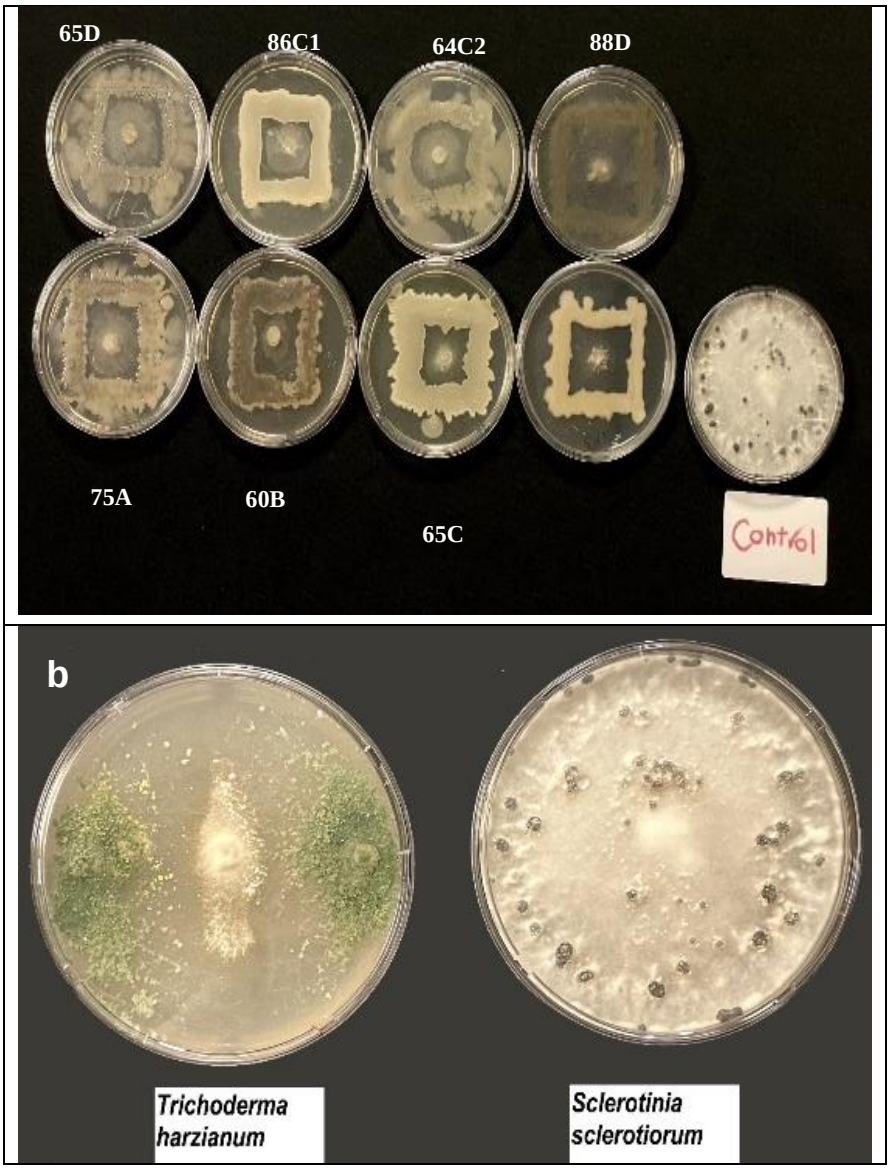

Fig. 7. In a dual culture on potato dextrose agar, *Bacillus* spp inhibited S. *sclerotiorum*. (a) dual culture of *Bacillus spp* and S. *sclerotiorum*, 20 °C, 4 dpi, demonstrating inhibitory zone between two colonies, *T. harzianum* inhibited S. *sclerotiorum*. (b) dual culture of *T harzianum* and S. *sclerotiorum*, 20 °C, 4 dpi, demonstrating inhibitory zone between two colonies.

After three days of incubation at 21°C, the eight strains of *Bacillus* spp. suppressed the mycelial growth of *S. sclerotiorum* in the range of 37% to 60% inhibition on PDA medium, compared to the control treatment without *Bacillus* spp. (Fig. 7a). Significant differences (p 0.001) were identified between antagonistic degrees of *Bacellus* isolates (65 D, 64 C2, 75 A138 C1, 88 D, 65 C, 60 B and 75 A). Duncan's multiple range test was applied to find treatments that differ significantly under various conditions of experimentation. S. *sclerotiorum* infection severity, which revealed significant virulence levels on PDA medium (Figure 8), was statistically examined. When compared to the positive control, the (65 D), (64 C2) and (75 A1), *Bacillus* isolates had the highest inhibitory effects against *S. sclerotiorum* (63%, 56 and 51%, respectively). The other bacterial isolates show inhibitory effects range from (50% to 39%) (Table 1). Significant differences in sensitivity to the eight strains of *Bacillus spp*. have been observed between *S. sclerotiorum* ($F = 4.36$, 218 df = 40, 82, $P < 0.0001$). The strains (65 D), (64 C2) and (75 A1), of *Bacillus* spp. highly inhibit the mycelial growth of *S. sclerotiorum* tested and the strains 38 C1, 88 D, 65 C, 60 B and 75 have a comparable inhibited the mycelial of *S. sclerotiorum* ($F = 4.36$, 218 df = 40, 82, $P < 0.001$). This inhibitory influence is really powerful (63% inhibition of mycelium growth) when cultured with 65 D, suggesting a strong antifungal effect of this strain of *Bacillus*

spp. tested (Fig. 9). Significant variations in *Bacillus* spp. strains have been found. ($F = 63.64$, $df = 9, 20$, $P < 0.0001$) with an inhibition ranging from 39 to 63% depending on the strain of *Bacillus* spp.

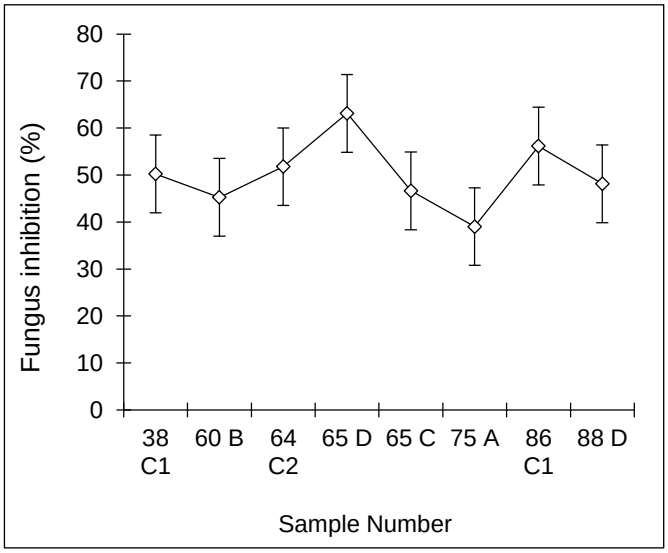

Fig. 8. Means (fungus inhibition (%).

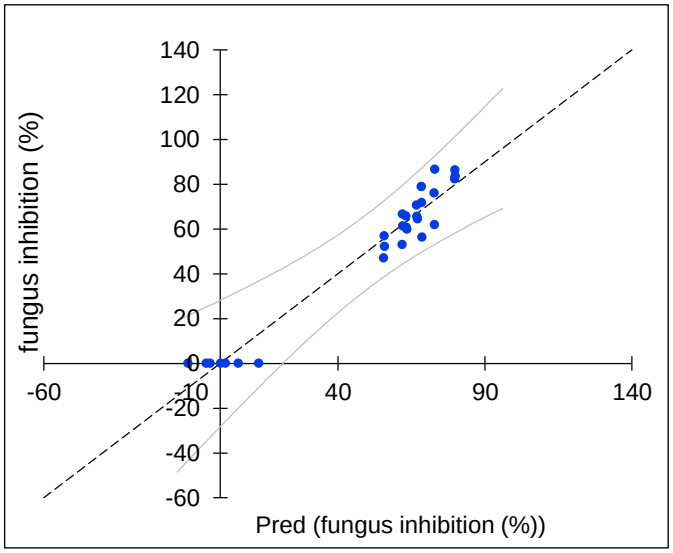

Fig. 9. Pred (fungus inhibition (%) /Standardized residuals.

Efficacy of treatments incorporating *Trichoderma harzianum*

For assessing mycelial growth, one disc containing inoculum of *S. sclerotiorum* was positioned at the center of Petri dishes, followed by the addition of two *Trichoderma harzianum*. discs on opposite sides of the plate. After 5-7 days' data on colony diameters were collected and utilized for mycelial growth analysis, analyses were conducted with a negative control without *Trichoderma harzianum*. *Trichoderma spp*. effectively reduced the growth rate of S. *sclerotiorum*, and total area of this fungus (Fig. 7b), indicating its potential as a biocontrol agent against this phytopathogen. The results from this study highlight the significance of biocontrol agents in combating various plant pathogens, potentially through specific antagonist-pathogen interactions.

4.0 Discussion

The infection of *S. sclerotiorum* can occur anywhere on the leaves but is most common on the stems and branches. Heffer and Johnson (2007) reported that the plant wilts completely if the infection starts at the base of the main stem, however, partial wilting will occur if the infection extends to the branches. When the inner part of the stem is exposed, fungal sclerotia filling the pith is observed. These sclerotia vary in size (small to large) and shape (elongated or cylindrical), often connecting to each other. They also form sclerotia on the main stem. White fungal mycelium sticking to the host's surface is also visible. The sclerotia range in color from brown to black (Iqbal *et al*., 2003). Morever, the flesh rots and a huge number of sclerotia can be seen in the decomposing tissues of fruits (Singh, 1987; Iqbal *et al.*, 2003).

S.sclerotiorum exhibit a higher average number of sclerotia per plate (Fig. 2 a). sclerotia showed a range of shapes (Fig. 2 c), typically varying from spherical to cylindrical, with sizes spanning from 2.0 to 20.0 mm (Jahan et al., 2022; Islam *et al.*, 2021; Prova *et al.*, 2018). These characteristics are comparable to those described for Sclerotinia (Hossain *et al.*, 2023). This result is in line with the findings of Cornwallis et al. (1993), who observed that around ± 10 days after inoculation (DAI), there was complete tissue collapse, resulting in the appearance of many plants as dead, despite the crown still exhibiting viability. Defoliation of leaves and the death of holl plant were observed as the disease progressed. The pathogen exhibited similar symptoms to those observed in naturally infected crops, affecting both uninjured and injured plants, although infection rates were higher in injured plants (Cheng *et al.*, 2019).

Nahar *et al.* (2019) found that the inculated eggplants with S. *sclerotiorum* with diferent isolates developed symptoms within 24 hours of inoculation and leaves discolored shortly after about a week. Pathogens were then extracted from sick tissues and grown on PDA plates before being characterized and the results revealed that they shared the same microscopic morphologies. (Li *et al.* 2008) identified significant variations in aggressiveness among isolates S. *sclerotiorum* collected from China, Canada, and England. Similarly, (Kull *et al.*, 2004) noted differing levels of aggressiveness among *S. sclerotiorum* isolates from various locations in North and South America. Moreover, in

Bangladesh, (Naher *et al.*, (2017) reported differences in pathogenicity among S. sclerotiorum isolates of mustard collected from different locations.

The molecular characterization of the 14 isolates through ITS sequencing confirmed their identification as *S. sclerotiorum* . Comparison of their ITS sequences with available sequences, such as KY750530, showed identical matches. Phylogenetic analysis based on these sequences demonstrated that the isolates clustered with publicly available *S. sclerotiorum* sequences, indicating their close relationship. They were found to be distinct from S. *rolfsii*, and *S. cepivora* (Figure 6). *S. sclerotiorum* are now the only fully sequenced species in the order Helotiales and with the obligate biotroph, in the class Leotiomycetes of the Pezizomycotina, the largest subphylum of Ascomycota (Spanu *et al.*, 2010). Tok et al., (2016) demonstrated the presence of significant heterogeneity within *S. sclerotiorum* populations and the coexistence of various isolates of the pathogen in the same regions through genetic diversity analysis using SSR and RAPD molecular markers in eggplant.

Due of their propensity to be endemic in the majority of regions, biological control agents are more environmentally friendly (Suriani *et al.*, 2020; Sukumawati *et al.*, 2021; *Kadiri et al.*, 2023; Paliwal et al., 2023). Antagonistic microbes have grown in soil, although this has not been associated with any discernible environmental harm (Cook and Baker 1983). The bacteria have demonstrated broad-spectrum efficacy against lettuce droplet *Sclerotinia* and the ability to produce a variety of antibiotics and mycolytic enzymes (Kamal *et al*., 2015a). The proportion of pathogen inhibition by rhizobacterial strains across the control was estimated using the formula given by (Yun, 2018) The antifungal activity of *Bacillus* spp via liposomal antibiotics and mycolytic enzymes was a direct result of the disintegration of fungal cell content and sclerotial cell organelles (Nithyapriya *et al.*, 2023; Krishna *et al.*, 2023; Sagar *et al.*, 2022; Kamal *et al*,. 2015; unpublished work). Given the advantages of antagonists, the development of commercial formulations with promising compounds may open the door to the long-term control of *S. sclerotiorum* in a variety of cropping systems, including those used for other vegetable crops.

5. Conclusions

The study investigates *Sclerotinia sclerotiorum*, a newly emerging fungal pathogen responsible for white mold disease in eggplant (*Solanum melongena* L.) in Saudi Arabia. It aims to understand the characteristics of this pathogen and evaluates several bio-agents' effectiveness in inhibiting its growth in laboratory conditions. Phylogenetic analysis showed that the *S. sclerotiorum* isolates clustered together with publicly available and were distinct from S. *rolfsii*, and *S. cepivora* groups. This study represents the initial evidence for exploring the morphological and genetic diversity of *S. sclerotiorum* in Saudi Arabia. Consequently, this report enhances our understanding of the disease's epidemiology and could aid in the development of effective disease management strategies.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: All the authors have consented for publication

Availability of data and material: All the data is included in the manuscript file. No data set was generated

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Authors' Contributions: Marwan A. Alsalamah, collecting samples, isolating and conducting host range studies, pathogenicity, and deposition of the isolates to the GenBank. Ayman F. Omar, characterize, identifying the fungus and conducted the DNA sequencing and phylogenetic analysis, Khalid E. Hamed, and R Z Sayyed. writing the manuscript reviewed, and edited. All authors have read and approved the final manuscript.

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List of Abbreviations

BCA -Biocontrol agent
DAI- Days after inoculation
FAO- Food and Agriculture order
ITS- Internal Transcribed Sequences
NA- nutrient agar
NB- Nutrient broth
PCR- Polymerase Chain Reaction
PDA -Potato Dextrose agar
rDNA - Ribosomal DNA

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