Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Molecular disparities among *Botrytis* species involved in onion umbel blight disease and its management using *Bacillus subtilis* PHYS7

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Abstract

A study of molecular variation among *Botrytis* spp., the causal pathogen of scape and umbel blights of onion, as well as the biocontrol of the virulent pathogen using *Bacillus subtilis* PHYS7 under greenhouse conditions was carried out. Twenty-three isolates of *Botrytis* spp. were recovered from onion plants showing umbel blight symptoms. They were able to infect onion plants with varied severity. All *Botrytis* spp. produced cellulases; however, almost all of them produced pectinase. The highest activities of cellulases and pectinase were achieved by *B. allii* PHYOA1; however, *B. cinerea* PHYOC3 showed a high activity of cellulases but it failed to produce pectinase. The genetic variability among the pathogens was assessed by random amplified polymorphic DNA (RAPD) markers, using 5 random 10-mer primers: OPA03, OPA05, OPA06, OPI09, and OPW15. The results showed that 50 DNA bands ranging from 100 bp (OPA03) to 1600 bp (OPA05) were generated by the 5 primers that differentiated 9 isolates of *Botrytis* spp. The dual culture test showed that *Bacillus subtilis* PHYS77 and PHYS78 had a high antagonistic potentiality against the pathogen and involved in 60–62% reduction in its growth. Application of Ridomil Gold MZ and *Bacillus subtilis* PHYS77 on onion plants, 2 days after or before infection with the pathogen, significantly reduced the disease severity than the control. The study approved the molecular tool as a reliable and quick method to differentiate among the virulent and non-virulent strains of *Botrytis* spp. The application of *B. subtilis* PHYS77 as an effective biocontrol agent in the management of the onion blight disease can be recommended.

Keywords: Onion umbel blight, Biological control, *Botrytis* spp., Cellulases, Pectinase RAPD markers

Background

Several fungal, bacterial, nematode, and viral diseases attack onion (*Allium cepa* L.) during all stages of its production season. Among the fungal diseases, *Botrytis byssoidea* and *B. allii* the causal pathogens of umbel blight of onion are destructive pathogens that involved in a great reduction in the productivity. The umbel blight disease is an important disease that attacks the plant during the production of the seeds and could devastate the unprotected crops up to 70% (Hussein et al., 2018). Spores of *B. byssoidea*, *B. allii*, *B. porri*, and *B. squamosa* cause infection for seedlings of green and bulb onions (Hussein et al., 2018). du Toit et al. (2004) reported the 4 diseases associated with onion seed crops causing blighting seed stalks and umbel and flower blight.

Molecular techniques based on the polymerase chain reaction (PCR) have been used as a tool in genetic mapping, evolutionary studies, molecular taxonomy, and diagnosis of several fungal species (Raja et al., 2017). Several workers have grouped *Botrytis* spp. population from different plant hosts using RAPD analysis and suggested that random amplified polymorphic DNA (RAPD) markers can be a reliable alternative for differentiating isolates of *Botrytis* spp.

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(Moyano et al., 2003). They reported that RAPD technology is suitable for studying the genetic structures of *B. cinerea* populations, generating and providing a better explanation of the genetic relationships among *B. cinerea* populations. This RAPD step is a powerful tool for grouping isolates and after more procedures can diagnose *B. cinerea* in plants, and may be useful to enhance knowledge of the epiphytology of the pathogen under field conditions (Rigotti et al., 2002). Although the management of these diseases depends on chemical fungicides, there are strong scientific demands to reduce the use of fungicides and the consequent environmental pollution and their residual toxicity (Alamri et al., 2019). Conducting environment-friendly control methods for the management of plant disease is a critical worldwide strategy (Abo-Elyousr et al., 2019). One of the promising trends in biocontrol of plant diseases is the application of antagonistic microorganisms (Abdel-Rahim and Abo-Elyousr 2018).

The objective of this study was to differentiate the molecular variations among different isolates of the causal pathogen of onion umbel blight disease *Botrytis* spp. by RAPD technique. The virulent isolates of the pathogen were bio-controlled by application of *Bacillus subtilis* PHYS77 under greenhouse conditions.

Materials and methods

Isolations of the causal pathogens

Twenty-three isolates of *Botrytis* spp. were isolated from naturally diseased onion plants showing symptoms of *Botrytis* Onion Umbel Blight (BOUB), collected from different onion seed producing farms, Assiut, Egypt (Hussein et al., 2018). Onion leaves, showing disease symptoms, were cut in small pieces, sterilized for 2 min in 2% sodium hypochlorite solution then, plated onto Potato Dextrose Agar (PDA) medium and incubated at 27 °C. After 4–5 days of incubation, the developing fungi were purified by a single spore technique on the same medium. The pure cultures of the isolated fungi were kept in refrigerator at 4 °C for further use.

Pathogenicity test

A pathogenicity test of *Botrytis* spp. isolates was carried out under greenhouse conditions. Inocula of the tested isolates were prepared by growing them on Petri plates (9-cm diameter) containing PDA medium at 25 °C for 15 days. Ten ml of sterile distilled water was added to each plate at the end of incubation period and the mycelial growth was carefully scraped with a sterile needle and used for inoculation of onion plants Cv Giza 6 that were grown in greenhouse at 20 ± 2 °C during the day and 18 ± 2 °C during the night. The onion bulbs were cultivated in pots (30-cm in diameter) filled with 5 kg sterilized sandy clay soil. Each pot was cultivated by one bulb of onion Cv Giza 6 and cared to grow for 120 days, then they were used for to pathogenicity test experiment (Abo-Elyousr et al., 2008). Five replicates were used for each *Botrytis* isolate. Onion flower heads "umbels" of the plants were inoculated by spraying with a spore suspension of the fungus at a concentration of 10^6 CFU/ml. Ten ml of the spore suspension was applied for each treatment (5 plants) using a hand atomizer. After inoculation, plants were covered with polyethylene cage for 48 h to provide high moisture, then polyethylene opened partially, after 72 h the polyethylene was removed. Plants were kept under normal conditions until the appearance of the symptoms. Disease severity was noticed after 14 days from inoculation. It was recorded as the average percentage of infected seed-head or umbel area and readings were converted to disease severity using the following equation:

$$\text{Disease severity}\% = [\Sigma\,(n \times V)/4 \times N] \times 100$$

where n = number of seed-head umbels within each infection category, N = total number of seed-stalk umbels examined, V = numerical values of infection categories, and 4 = constant, highest numerical value.

Scales of 0–4 were used by Hussein et al. (2007) as follows:

0 = No infection, 1 = 25% of seed-head umbel infected area, 2 = 50% of seed-head umbel infected area, 3 = 75% of seed-head umbel infected area, 4 = 100% of seed-head umbel infected area.

Identification of the causal pathogens

Morphological identification

Morphological characteristics of mycelia and spores were used to identify the fungal isolates following identification references (Ellis and Waller, 1974; Yohalem et al., 2003; du Toit et al., 2004 and Chilvers and du Toit, 2006) with aid of mycologist of Assiut University Mycological Center, Assiut, Egypt.

Identification of Botrytis spp. using polymerase chain reaction-restriction fragment length polymorphism

PCR detection and restriction fragment length polymorphism (RFLP) were used for identification of *Botrytis* spp. isolated from the infected onions. The following DNA primers for amplification and subsequent identification of 4 different *Botrytis* spp. were used, using sequenced characterized amplified regions (SCARS): BA1r: 5′-TGAGTGCTGGCGGAAACAAA-3′ and BA2f: 5′-GTGGGGGTAGGATGAGATGATG-3′.

Using a Polymerase Chain Reaction (PCR) assay, the primers amplify around 413 bp DNA product from species. Restriction fragment length polymorphism (RFLP) analysis of the PCR amplicon was carried out using the restriction enzyme ApoI and 500 bp ladder (New England Biolabs, Beverly, MA) (Nielsen et al., 2002).

Variations among the tested isolates of the causal pathogen

Cellulases productivity

Detection of exo-1.4 ß-glucanase (C1)

The ability of the pathogenic isolates to produce exo-1, 4 ß-glucanase (C1) was screened. The tested fungi were inoculated into 250 ml Erlenmeyer's conical flasks containing 50 ml of cellulase production medium, which had the composition: (g/l) L-asparagine 0.5, yeast extract 0.5, $(NH4)_2SO_4$ 0.5, KCL 0.5, KH_2PO_4 1.0, $CaCl_2$ 0.2, $MgSO_4.7H_2O$ 0.2, and cellulose microcrystalline 10, as described by Luo et al. (1997). The pH was adjusted to 7.0, using acetate buffer and the medium was incubated at 28 ± 1 °C for 7 d under static conditions. The growing medium was filtrated, using a filter paper Whatman No 1, the mycelial mats were excluded, and the filtrate was considered as a crude enzyme. A well (0.5 cm in width and 0.4 cm in depth) in the agar medium (had the same composition as mentioned above) was filled with 100 μl of the filtrated crude enzyme, and the plates were incubated at 28 °C for 24 h. Then, the plates were flooded with 1% iodine solution (1 g iodine + 3 g KI + 100 ml water) and shaken for 5 min, the iodine solution was decanted. The clear zone around wells indicated positive for cellulase production by the releasing exo-1, 4-β-glucanase (C1). The activity of the enzyme was identified by measuring the diameter of the clear zone around the wells in mm.

Detection of endo-1.4 ß-glucanase (Cx)

Twenty-three isolates of the pathogen were screened for their ability to produce endo-1, 4 ß-glucanase (Cx) according to (Oksanen et al., 2000).

Pectinase productivity

The method was carried out as described by Hankin et al.(1971). The used medium contained the following constituents, which were prepared in 2 main portions (g/l):

Portion A: Yeast extracts (1 g), pectin from citrus peel (5 g), Agar (15 g), distilled water (500 ml), and its pH was adjusted to 7.0. **Portion B:** Mineral salt solution composition (per liter) of: KH_2PO_4 (4 g), $(NH_4)_2SO_4$ (2 g), Na_2HPO_4 (6 g) $CaCl_2$ (1 mg), $CuSO_4$ (50 μg), H_3PO_4 (10 μg), $MnSO_4$ (10 μg), $ZnSO_4$ (70 μg), $FeSO_4.7H_2O$ (1.0 mg), MoO_3 (10 μg), distilled water (500 ml) and pH was adjusted to 7.4.

The two portions were mixed thoroughly after autoclaving at 121 °C for 15 min. The pathogenic isolates were cultured into 250 ml Erlenmeyer's conical flasks containing 50 ml broth medium for each and incubated at 28 °C for 7 days. A medium with the same composition (as above) solidified with agar was dispensed into 9-cm Petri dishes (20 ml per plate); and the Petri dishes were then inoculated with the crude enzyme filtrate in 1 cm diameter wells and incubated for 24 h at 28 °C. After the incubation period, the dishes were flooded with 1% iodine solution, the excess of iodine was poured off from the plates. The appearance of the clear zone around wells indicates the production of pectinase enzyme (Ammar et al., 1995). The activity of the enzyme was evaluated by measuring the clear zone around the wells in mm.

Molecular dissimilarities among the causal pathogens using random amplified polymorphic DNA

1. Cultivation of the fungal strains for DNA isolation

The pathogenic fungi were inoculated into 50 ml of potato dextrose broth in 250-ml flasks. The flasks were incubated for 7 d at 27 °C, and the medium was filtered through a Buchner funnel after incubation, the mycelia were washed thoroughly twice with sterilized distilled water. Hundred mg of the obtained mycelia was used for DNA isolation as described by Cenis (1992).

2. Random amplified polymorphic DNA of *Botrytis* spp.
   Nine isolates of *Botrytis* spp. were selected for amplification of the random polymorphic DNA. The 9 isolates were selected out the 23 isolates based on their source and their disease severity (low, medium and high). The selected isolates were *B. squamosa* PHYOQ 1, *B. allii* PHYOA1, *B. allii* PHYOA3 *B. allii* PHYOA5 *B. allii* PHYOA7, *B. cinerea* PHYOC2, *B. cinerea* PHYOC3, *B. aclada* PHYOL1 and *B. aclada* PHYOL2**.** PCR amplification of genomic DNA was performed using 5 arbitrary primers set (Table 1) purchased from Operon Technologies (Alameda, CA). Amplification reactions were carried out in 25 μl reaction volumes containing 40 ng of purified fungal DNA and 10 pmol of primer. The reaction mixture contained 400 μM of each dNTP, 2.5 mM $MgCl_2$, 1x reaction buffer and 1 unit of DNA polymerase (BioFlux cat. BSA12M1). The volume was adjusted to 25 μl by the addition of sterile distilled water. Amplification was performed in a MJ research PTC-200 thermal cycler, with an initial

Table 1 Sequences of 10 mer arbitrary primers used in RAPD-PCR

No.	Primer name	Sequence 5′ → 3′
1	OPA03	AGTCAGCCAC
2	OPA05	AGGGGTCTTG
3	OPA06	GGTCCCTGAC
4	OPA09	GGGTAACGCC
5	OPA15	TTCCGAACCC

denaturation at 94 °C for 4 min, followed by 35 cycles each of denaturation at 94 °C for 60 s, annealing at 35 °C for 60 s and extension at 72 °C for 120 s. A final extension at 72 °C for 7 min, then samples were cooled and held at 4 °C until use. PCR products were loaded into a 1.5% agarose gel in 1× TE containing ethidium bromide at 100 volts, constant voltage for 3 h. The electrophoresis gels were observed and photographed under a UV transilluminator by using a Herolab gel-documentation system model Mididoc (Herolab, Germany).

Biological control of the disease under greenhouse conditions

1. Source of the bioagents

Five bacterial isolates, *Bacillus subtilis* PHYS77, *B. subtilis* PHYS78, *B. subtilis* PHYS79 and *B. subtilis* PHYS80, *Pseudomonas fluorescence* PHYPf were obtained from Plant Pathology Department, Faculty of Agriculture, Assiut University, Assiut, Egypt, and were tested to determine their antagonistic capability against *B. allii* PHYOA1 (the most virulent isolate).

2. Antagonistic effect of bacterial isolates in vitro

The bacterial bio-agents were screened under in vitro against *B. allii* PHYOA1 for their antagonistic activities, using dual culture method as described by Abo-Elyousr and Mohammed (2009).

3. Pot experiments

Pot experiments were carried out in a greenhouse and the inoculation of the pathogen within the plants was carried out as mentioned in pathogenicity test. *B. subtilis* PHYS77 was grown on NA medium in conical flasks each containing 100 ml NA medium and incubated at 28 °C for 2 days. The cultures were centrifuged at 1500 rpm for 15 min. The optical density OD_{600} nm of the *B. subtilis* PHYS77 suspension was adjusted to obtain 4 × 10^7 CFU/ml, water. Ten ml of the suspension was sprayed on onion plants, using a hand atomizer (Sallam Nashwa et al., 2009). Disease severity percentage was noticed after 14 d from inoculation according to Hussein et al. (2007). The fungicide "Ridomil gold MZ, mefenoxam 6% + copper oxide 60%," at a concentration of 0.2% was used as a positive control.

Statistical analysis

Both greenhouse and lab experiments were carried out with 4 replicates/ treatment and the experiments were repeated twice. The laboratory treatments were arranged in a complete randomized design, while the greenhouse treatments were arranged in a completely randomized plot design. Analysis of variance was done, and the significance of differences among the treatments was determined according to the least significant difference (LSD) $P < 0.05$ (Gomez and Gomez, 1984).

Results and discussion

The causal pathogen of BOUD

Occurrence of *Botrytis* onion umbel blight disease in Assiut, Egypt, was studied throughout the survey of many onion seed production areas. From diseased onion floral umbels, 23 fungal isolates were recovered. The results indicated that onion plants were heavily infected with the fungal pathogens. Recent studies reported that the cultivated area all over the world decreased as a result of infections by several pathogens that attack onion during growing seasons (Schwartz and Mohan, 2008). Five species of *Botrytis* were associated with bulb rots of onion and neck, umbel and affected onion crop productivity (Lorbeer et al., 2007). *Botrytis allii*, *B. aclada*, *B. cinerea*, *B. byssoidea*, and *B. squamosa* were described as the cause of sclerotial neck rot, mycelial neck rot, small sclerotial neck rot, and *Botrytis* Onion Umbel Blight (Langston and Saunders, 2009). Blighting of the umbels of onions by *Botrytis* spp. was reported in Egypt by Abd-El-Razik et al. (1977). Also, Langston and Saunders (2009) reported that *Botrytis squamosa*, *B. cinerea*, and *B. allii* were involved in the flower blight of onion (*Allium cepa* L.).

Identification of the causal pathogens

Morphological identification

Identification procedures of the 23 fungal isolates, recovered from naturally diseased onion heads, showing *Botrytis* Onion Umbel Blight (BOUB) symptoms approved their classification as members of the genus *Botrytis* (Table 2) to confirm their association with the onion diseases. The 23 isolates belong to 4 species of *Botrytis* spp. *Botrytis allii* was represented by 13 isolates. Seven isolates were identified as *B. cinerea* and 2 isolates belonged to *B. aclada*, whereas *B. squamosa* was represented by one isolate. Higher isolation frequencies of both *B. allii* (56.5%) and *B. cinerea* (30.5%) confirm their potential as the main causal pathogens of the onion flower disease. The results are in agreement with Ellerbrock and Lorbeer (1977), who reported that *B. cinerea* and *B. allii* were isolated from flower blight, and they are the main causal agents for many diseases of onion flower as well as the findings of Langston and Saunders, 2009These species were frequently associated with onion neck rot (Chilvers and du Toit, 2006; Hafez et al., 2013 and Sayed et al., 2014), scape blight and bulb rot (Chilvers and du Toit, 2006), Botrytis leaf blight (Brankica Tanović et al., 2019), and leaf blast (Segall and Newhall, 1960).

Table 2 Pathogenic capabilities of *Botrytis* spp. isolates on onion cultivar Giza 6 under open greenhouse conditions

Isolate no.	Source of isolate	Pathogens	Disease severity (%)
1	Elfath, Assiut	*B. allii* PHYOA1	91.7
2	ElZawia, Assiut	*B. allii* PHYOA2	65.0
3	Reefa, Assiut	*B. allii* PHYOA3	58.9
4	Abo-Teeg, BniAdii, Assiut	*B. allii* PHYOA4	41.1
5	Abo-Teeg, Assiut	*B. allii* PHYOA5	77.8
6	Manfalout-ElAtamna, Assiut	*B. allii* PHYOA6	75.6
7	Manfalout	*B. allii* PHYOA7	25.0
8	Manfalout	*B. allii* PHYOA8	58.3
9	Reefa, Assiut	*B. allii* PHYOA9	46.7
10	Dirout, Assiut	*B. allii* PHYOA10	50.0
11	Durunka, Assiut	*B. allii* PHYOA12	71.7
12	Mangabad, Assiut	*B. allii* PHYOA12	52.2
13	Alghanaim, Assiut	*B. allii* PHYOA13	67.2
14	Abnoob, Assiut	*B. cinerea* PHYOC1	50.0
15	Mousha, Assiut	*B. cinerea* PHYOC2	66.7
16	Assiut	*B. cinerea* PHYOC3	83.3
17	El-Koussia, Assiut	*B. cinerea* PHYOC4	27.8
18	ArabElAwamer, Assiut	*B. cinerea* PHYOC5	47.2
19	Assiut Valley	*B. cinerea* PHYOC6	44.4
20	ArabElAwamer	*B. cinerea* PHYOC7	58.3
21	Dirout, Assiut	*B. aclada* PHYOL1	40.0
22	Abnoob-Elhamam, Assiut	*B. aclada* PHYOL2	33.3
23	Assiut Valley	*B. squamosa* PHYOQ 1	22.2

Least significant difference LSD ($P < 0.05$) = 6.18

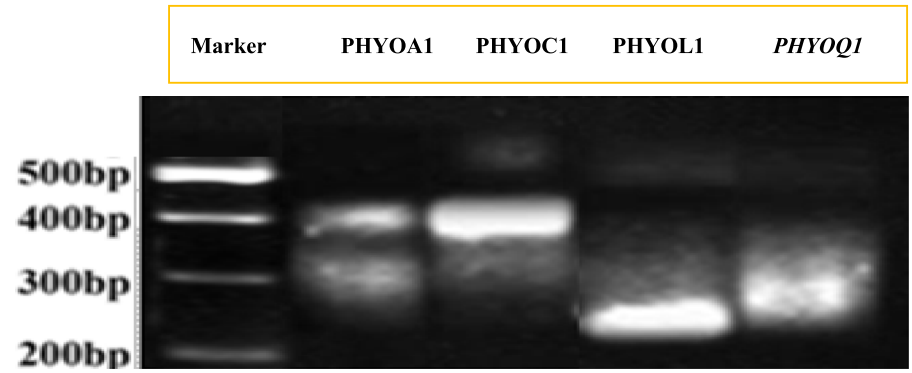
Molecular identification of the fungal pathogens using PCR-RFLP

Application of PCR-RFLP was carried out, using the restriction enzyme ApoI and specific primers amplified an amplicon of *Botrytis* DNA, which produced 400 bp. This specific amplification was diagnostic and different among the 4 *Botrytis* spp. (Fig. 1). It was shown that *B. squamosa* had a specific band with 269 bp, *B. aclada* has a 413-bp band, *B. allii* has 2 bands 413 bp and 298 bp, and *B. cinerea* has a 250-bp band. The molecular characterization, using PCR-RFLP fitted well with the morphological characterization of the 4 species of *Botrytis.* These results matched the previous morphological identification and matched well with the results mentioned by Hafez et al. (2013).

PCR-RFLP is highly reproducible which makes it possible to predict genetic parameters more accurate and labor intensive (Raja et al., 2017). Previous reports on onion diseases caused by *Botrytis* spp. have not differentiated between *B. aclada* and *B. allii*, making it difficult to determine the relative distribution and significance of these 2 species as neck rot pathogens. However, since description of PCR and PCR-RFLP assay by Nielsen et al. (2002) for the primary neck rot *B. allii*, *Botrytis* spp. and *B. aclada* can be differentiated more readily; also Nielsen et al. (1999) collected 29 isolates from different countries and identified them morphologically as *B. aclada* or *B. allii*; however, when they applied the PCR-RFLP assay, they were separated into 3 species: *B. byssoidea*, *B. squamosa*, and *B. allii* (Nielsen et al., 2001 and Chilvers et al., 2004).

Pathogenicity test

Results indicated that all tested isolates of *Botrytis* spp. were able to infect onion plants causing *Botrytis* umbel blight disease (BOUB) with varied disease severity (Table 2). *Botrytis allii* PHYOA1, *B. cinerea* PHYOC3, *B. allii* PHYOA5 and *B. allii* PHYOA6 were involved in the highest disease severity (91.7, 83.9, 77.8, and 75.6 %, respectively), while *B. squamosa* PHYOQ5 and *B. allii* PHYOA7 exhibited the lowest disease severity on onion umbels (22.2–25.0%). The other isolates of the 4 species of *Botrytis* exhibited moderate severity of BOUB disease, ranged between 27.8–67.2%. The variation in disease severity and virulence of the pathogens could be affected by climatic conditions in regions, where they were isolated. In a



PHYOA1= *B. allii* PHYOA, PHYOC1 = *B. cinerea* PHYOC1, PHYOL1= *B. aclada* PHYOL1, PHYOQ1 = *B. squamosa* PHYOQ1

Fig. 1 PCR-RFLP analysis of four *Botrytis* spp. isolated from onion infected umbels

previous study, Shoemaker and Lorbeer (1977) reported that the free moisture is necessary for infection of umbel blight of onion by *B. allii*, *B. cinerea*, and *B. squamosa*. Similarly, Brankica Tanović et al. (2019) observed that temperature and moisture are the main factors that affect the pathogenicity of *Botrytis* spp. on the umbel of onions.

Variations in enzyme activity among the tested Botrytis spp.

Cellulase production The results indicated that most potent isolates were *B. allii* PHYOA1, *B. allii* PHYOA5, *B. cinerea* PHYOC3, and *B. cinerea* PHYOC2, whereas they showed the highest level of Exo-1, 4-β- glucanase (C1) on cellulase production medium. The enzyme activity of the 4 organisms was measured as 16-18 mm (Table 3). The rest of the fungal isolates showed a moderate to low activity (up to 12 mm of clear zone). *Botrytis allii* PHYOA1, *B. allii* PHYOA 5, *B. allii* PHYOA11, *B. cinerea* PHYOC3, and *B. cinerea* PHYOC7 were recorded as good producers of endo-1,4-β-glucanase (Cx), where the clear zone ranged from 22 to 18 mm on cellulase production medium, while the other isolates showed low activity of endo-1,4-β-glucanase (Cx) (Table 3). Interestingly, *B. allii* PHYOA1 was among the isolates having the capabilities to produce a high amount of cellulase enzymes, involved in high disease severity on onion plants. The fungal isolates, which were characterized by moderate or low virulence on onion plants, showed a low productivity of cellulase. The assumption that disease severity of the pathogen could be related to its capacity to produce cellulases was supported by the findings of Chen et al. (2018).

Pectinase production The results indicated that *B. allii* PHYOA1 and *B. allii* PHYOA5 had the highest activity

Table 3 Cellulases and pectinase production of *Botrytis* species of onion *in vitro*

Isolates	Cellulases (mm)		Pectinase (mm)
	C1	Cx	
B. allii PHYOA1	18*	22	19*
B. allii PHYOA2	12	16	10
B. allii PHYOA3	10	14	-ve
B. allii PHYOA4	10	16	13
B. allii PHYOA5	16	22	15
B. allii PHYOA6	14	18	6
B. allii PHYOA7	10	10	11
B. allii PHYOA8	12	14	8
B. allii PHYOA9	12	12	-ve
B. allii PHYOA10	12	12	7
B. allii PHYOA11	12	20	17
B. allii PHYOA12	14	18	10
B. allii PHYOA13	14	16	6
B. cinerea PHYOC1	14	16	10
B. cinerea PHYOC2	16	16	11
B. cinerea PHYOC3	18	22	-ve
B. cinerea PHYOC4	12	12	12
B. cinerea PHYOC5	12	12	4
B. cinerea PHYOC6	14	12	9
B. cinerea PHYOC7	14	18	13
B. aclada PHYOL1	16	16	15
B. aclada PHYOL2	12	12	14
B. squamosa PHYOQ 1	12	12	17

*Cleared zone caused by the isolate on the medium was measured in mm

of pectinase production, while *B. allii* PHYOA6 and *B. allii* PHYOA13 had the lowest production (Table 3). On the other hand, both *B. allii* PHYOA3 and *B. allii* PHYOA9 did not produce any detectable amounts of pectinase. *Botrytis cinerea* PHYOC4 and *B. cinerea* PHYOC7 had high activities of the enzyme, while *B. cinerea* PHYOC5 showed low one. The other strains produced considerable amounts of the enzyme than with *B. allii* PHYOA11. Obtained results showed that the productivity of pectinase by the fungi was correlated by their pathogenicity. Both *B. allii* PHYOA1 and *B. squamosa* PHYOQ1, which were characterized by their high activities of pectinase, were in a high disease severity on onion plants.

Polymorphism among *Botrytis* spp. causing BOUB disease using RAPD RAPD technique requires single oligonucleotides that able to produce multiple bands, using primers that can be used to generate a large number of fragments from different regions of the genome and multiple loci can be examined very quickly. Polymorphism and genetic similarity among 9 *Botrytis* spp., using 5 random 10-mer primers (OPA03, OPA05, OPA06, OPI09 and OPW15) were analyzed to investigate the genetic differences among pathogenic *Botrytis* spp. generated 50 DNA bands (Fig. 2) ranging from 100 bp (OPA03) to 1600 bp (OPA05). The genetic similarity (GS) among the species was analyzed, using the software package MVSP program (Nei and Li, 1979). The genetic similarity among the nine isolates of *Botrytis* spp. ranged from 66.7 to 87.8%. The highest similarity and shortest genetic distance were scored between *B. allii* PHYOA1 and *B. allii* PHYOA5; however, the lowest genetic similarity was found between *B. squamosa* PHYOQ1 and *B. cinerea* PHYOC2 (Fig. 2 and Table 4). The dendrogram tree of the nine isolates of *Botrytis* spp. resulting from the UPGMA clustering values, illustrated in Fig (2) and Table (4) revealed the relationship of the species (Fig. 3) based on the number of markers that were different between any given pair isolates. Cluster dendrogram based on similarity matrix and obtained with unweighted pair group method, using arithmetic means (UPGMA) showed that the 9 *Botrytis* spp. had a high similarity value among each other (0.706 to 0.878) (Fig. 3). Data indicated that *B. allii* PHYOA1 and *B. allii* PHYOA5 displayed the highest number of DNA fragments (43 bands), while *B. cinerea* PHYOC2 revealed the least number of bands (29 bands). These variations in the number of bands, amplified by different primers, were influenced by variable factors such as primer structure and number of annealing sites in the genome. Polymorphic bands were detected by all primers surveyed in all isolates of *Botrytis* spp. Out of 71 DNA-bands, 23 were conserved among all isolates tested, while 45 (63.3%) were polymorphic. The monomorphic bands were constant bands and cannot be used to study the diversity, while polymorphic bands revealed differences and could be used to examine and establish systematic relationships among the genotypes.

Based on molecular markers with the aid of the RAPD–PCR technique, *Botrytis* spp. involved in infection of onion blight were easily differentiated. Such information may lead to the development of more reliable methods for early detection of pathogens in the field (Paplomatas et al., 2004). Generally, a total of 71 DNA bands ranged from 100 bp (OPA03) to 1600 bp (OPA15) were generated by the 5 primers for the 9 isolates of *Botrytis* species. From the 71 bands obtained, 45 were polymorphic that revealed differences among the species and could be used to examine and establish systematic relationships among the genotypes. The highest number of amplified DNA fragments was detected for the primer OPA05, while the lowest number was amplified with the primer OPA05. *B. allii* PHYOA5 displayed the highest number of DNA fragments, while *B. cinerea* PHYOC2 revealed the least number of bands. Related previous work by Kerssies et al. (1997) who studied 29 isolates of *Botrytis cinerea* for correlation between their markers and pathogenicity and collected from inside and outside Dutch glasshouses, demonstrated that from 70 markers scored, few of them showed a high similarity. Cluster analysis recognized three groups. The presence of a unique band for a given genotype was taken as a positive marker, while the absence of a unique band referred as a negative marker. Such bands could be used as DNA markers for isolate identification and discrimination.

In this respect, the OPA03 primer showed specific positive bands for *B. allii* PHYOA1 and *B. allii* PHYOA5 that had the highest disease severity. This primer did not produce any detectable bands with *B. squamosa* PHYOQ1, *B. cinerea* PHYOC2 and *B. aclada* PHYOL2. Primer OPA09 produced specific positive bands for *B. allii* PHYOA3 with molecular weight (193 bp) and negative band at molecular weight 256 bp. Primer OPA06 showed two negative specific bands for *B. allii* PHYOA7 with molecular weight 347 bp and 454 bp and 2 positive specific bands with molecular weights of 411 and 175 bp, respectively. Primer OPA15 produced one positive and one negative specific band for *B. allii* PHYOA5 with molecular markers (326 and 534, respectively). These findings are significantly similar to those obtained by Martinez et al. (2003).

Most of RAPD-PCR specific markers were scored for isolate *B. squamosa* PHYOQ1, *B. allii* PHYOA1

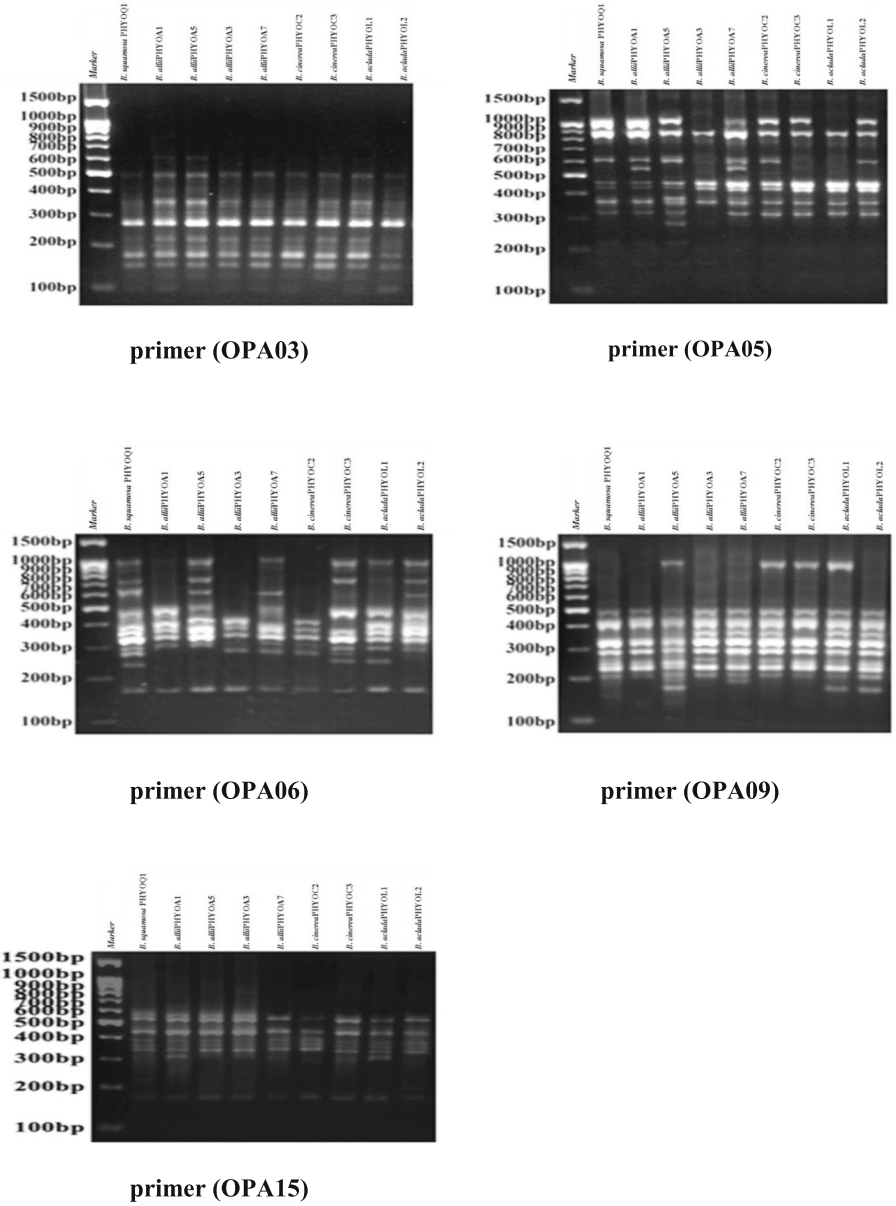


Fig. 2 Agarose gel electrophoresis of RAPD-PCR primers OPA03, OPA05, OPA06, OPI09 and OPW15 profile of nine *Botrytis* spp. isolates

and *B. allii* PHYOA7. Obtained results indicated that 9 isolates-specific markers (7 positive and 2 negative) were detected and they could be used as markers for the investigated species. This study showed that there was a considerable genotypic variability among isolates of the 4 *Botrytis* spp. that were obtained from different geographic regions. This variation could be due to variations in geographical regions, where the climates are different or due to the disease severity of the isolates, which could be controlled by specific genes. These results are in line with those of Isenegger et al. (2008). The later assumption may be supported by the finding of Thompson and Latorre (1999) who mentioned that no relationship was found between geographical origins and the DNA profiles obtained from the study of *B. cinerea* in Chile. These results encourage to carry out further investigation on the molecular level to achieve more specific characterizations of the *Botrytis* spp. Our hypothesis is that the DNA fragment with molecular specific size can appear only in one unique isolate and can display high disease severity percentage and such band may be closely related to high virulence or be a distinguisher to specific pathogen.

Table 4 Genetic similarity values calculated from total DNA fragments generated with five primers in nine *Botrytis* spp.

Similarity matrix	*B. squamosa* PHYOQ1	*B. allii* PHYOA1	*B. allii* PHYOA5	*B. allii* PHYOA3	*B. allii* PHYOA7	*B. cinerea* PHYOC2	*B. cinerea* PHYOC3	*B. aclada* PHYOL1	*B. aclada* PHYOL2
B. squamosa PHYOQ1	1								
B. allii PHYOA1	0.706	1							
B. allii PHYOA5	0.761	0.774	1						
B. allii PHYOA3	0.675	0.744	0.753	1					
B. allii PHYOA7	0.723	0.786	0.725	0.711	1				
B. cinerea PHYOC1	0.737	0.701	0.714	0.783	0.8	1			
B. cinerea PHYOC3	0.729	0.744	0.753	0.692	0.738	0.753	1		
B. aclada PHYOL1	0.667	0.756	0.719	0.703	0.775	0.74	0.878	1	
B. aclada PHYOL2	0.78	0.747	0.756	0.693	0.79	0.757	0.747	0.759	1

Biological control of the BOUB disease

Antagonistic effect of bacterial isolates in vitro Results of in vitro test (Fig. 4) showed that the 3 tested bacteria reduced the mycelial growth of *B. allii* PHYOA1. The highest inhibition percentage was scored by *B. subtills* PHYS77 and *B. subtills* PHYS78 (62 and 60%, respectively), followed by *B. subtilis* PHYS79 and *B. subtilis* PHYS80 (48 and 46%), while *Pseudomonas fluorescence* recorded the lowest inhibition percentage of the mycelia growth PHYPf1 (45%). According to the above results, the isolate PHYS77 was selected for greenhouse experiment. The biological control capacity of *Bacillus* spp. was reported because produced several antibiotics having a broad range of fungal inhibition. Antibiotic production as a mode of action of *Bacillus* pp. was established and reported in previous studies (Alamri et al., 2019).

Suppression of disease severity of BOUB under greenhouse conditions The results indicated that antagonist *B. subtilis* PHYS77 reduced the disease severity, when it was applied before or after infection with the pathogen to 64.7 and 60.1%, respectively compared to 94.4 0% resulted from chemical fungicide (Ridomil Gold MZ) application under greenhouse conditions (Table 5). The results obtained here were very promising to apply the bacterial strains as a biological agent alternative to the fungicides in the management of such disease as a single application or as a part of the integrated management program (IPM). In accordance with our results, *Botrytis* spp. the causal pathogens of BOUB disease, was managed biologically

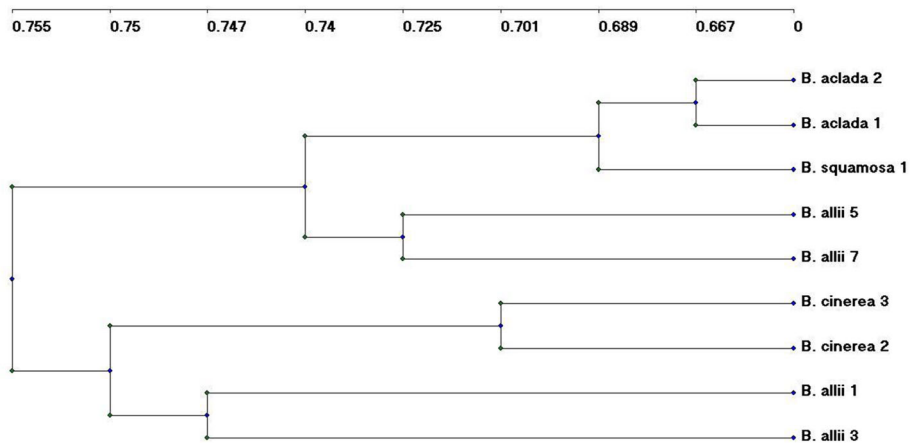


Fig. 3 Dendrogram developed from PCR-RAPD five primers using UPGMA analysis. The scale is based on Nei and Li coefficients of similarity

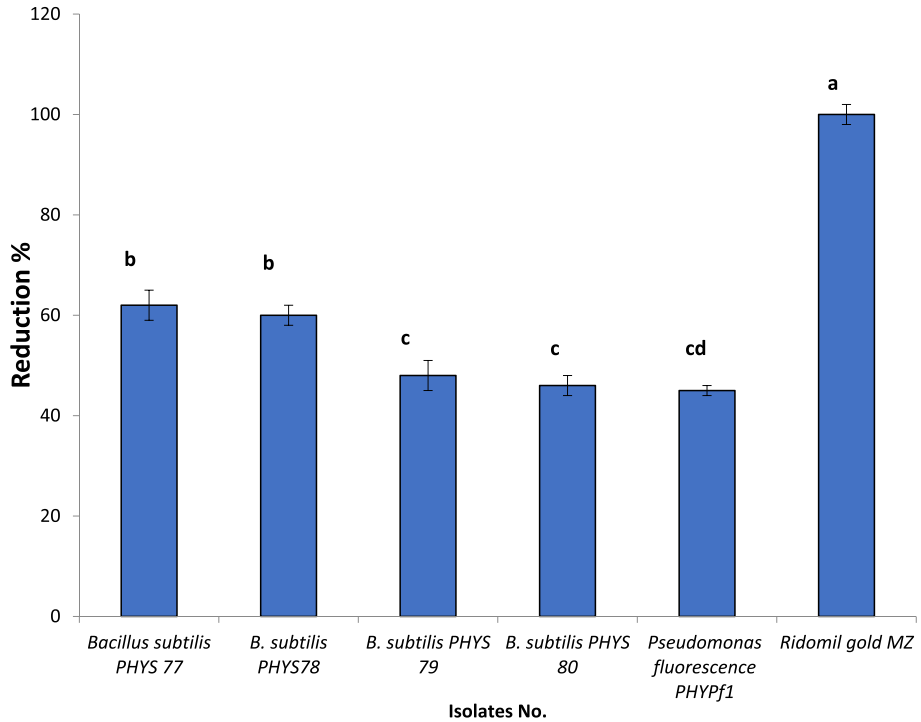


Fig. 4 Evaluation of inhibitory effect of certain bacterial isolates against *B. allii* PHYOA1 *in vitro*. Values on the column followed by a similar letter are not significantly different as determined by the LSD test ($P < 0.05$)

by different antagonistic bacteria (Abo-Elyousr et al., 2014). *B. subtilis* as a good antagonistic microorganism was reported by its variable mechanisms of action against many fungal pathogens (Alamri et al., 2019; Punja et al., 2019 and Grata et al., 2019). *B. subtilis* produces bacteriocins that play an important role in essential host immunity (Hashem et al., 2019). Also, it may have indirect mechanisms against plant pathogens including, competition for nutrients, plant growth promotion, biofilm formation, and colonization sites, induced systemic resistance (ISR) (Wang et al., 2018). Activation of the induced resistance in plants by *B. subtilis* was known before (Garcia-Gutierrez et al., 2013) via induction the synthesis of ethylene and jasmonic acid (JA). Other strains belonging to the *Bacillus* spp. were well documented to produce an auxin (IAA) (Abo-Elyousr et al., 2019) and can retain more soil organic nitrogen and other nutrients in the plant. It has also been known to produce compounds, which promote plant growth directly or indirectly.

Conclusion

The study concludes that *Botrytis* onion umbel blight (BOUB) is a complex disease that could be caused by many pathogens as at least 4 *Botrytis* spp. were identified. Results suggest that application of RAPD technique by using specific primers can be utilized as a simple and quick diagnostic technique for pre-diagnosis of onion infection with *Botrytis* spp. to avoid their destructive effect at the flowering stage. As an ecofriendly management of the BOUB, the study recommends the application of the bioagent *B. subtilis* PHYS77 to reduce the disease severity and protect the plants during the growing season.

Table 5 Effect of treatment with *Bacillus subtilis* PHYS77 on disease severity % of BOUB caused by *B. allii* PHYOA1 under greenhouse conditions

Treatment	Treatments before infection		Treatments after infection		
	Disease severity (%)	Disease reduction (%)	Disease severity (%	Disease reduction (%)	Disease severity (mean %)
Bacillus subtilis PHYS77	31.7 b	64.7	35.0 b	60.1	33.35 b
Ridomil gold MZ	5.00 c	94.4	5.00 c	94.3	5.00 c
Infected control	90 .0 a	0.00	87.8 a	0.0	88.9 a

Values in the column followed by a similar letter are not significantly different as determined by the LSD test ($P < 0.05$)

Acknowledgments
The authors would like to express their gratitude to the Research Center of Advanced Materials - King Khalid University, Saudi Arabia, for the support through grant number RCAMS/KKU/002-19.

Authors' contributions
KA participated in the planning and designing of the experiments, implementation of the experiments, and writing of the manuscript. AS bacterial identification, analyzing data. MH participated in the planning and implementation of the experiments, sampling, lab work, and writing the draft. MH participated in the planning and implementation of the experiments and writing the draft and AB participated in the implementation of the experiments and sampling. MH lab work and collecting data and revised the manuscript. All authors read and approved the final manuscript.

Funding
This study was financially supported by the Research Center of Advanced Materials - King Khalid University, Saudi Arabia through grant number RCAMS/KKU/002-19.

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
This manuscript is in accordance with the guide for authors available on the journal's website. Also, this work has not been published previously and is approved by all authors and host authorities.

Consent for publication
All authors approved the publication of the study.

Competing interests
The authors declare that they have no competing interests.

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Received: 19 November 2019 Accepted: 5 January 2020
Published online: 14 January 2020

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