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Postharvest diseases of tomato and natural products for disease management

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Gray mold and soft rot are the most important postharvest diseases of tomato worldwide. A survey of fresh-market tomato fruit was conducted in Oahu to determine which fungal and bacterial pathogens were most commonly associated with postharvest disease. *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Mucor*, *Stemphyllium*, *Rhizopus* and *Penicillium* were the most frequently isolated fungi and *Acetobacter*, *Gluconobacter*, *Klebsiella*, *Leuconostoc* and *Pectobacterium* were the prevalent bacteria. Fifty-one percent of the diseased tomatoes had been imported from California and Mexico and 49% had been grown locally at three sites in Oahu. Pathogenicity tests revealed that 33 of 99 fungal isolates and 10 of 17 bacterial isolates were pathogenic on tomato types known as common market, cherry and grape tomato. Based on fruit assays, *Botrytis cinerea* (B03) and *Pectobacterium carotovorum* (BA17) were the most virulent isolates. Tested leaf extracts of *Capsicum annuum* cv. Stocky Red, *C. annuum* cv. Criolla de cocina, *Capsicum chinense* cv. NuMexsuave, *Tagetes tenuifolia*, *Aloe vera*, *Origanum vulgare* and *Azadirachta indica* were ineffective as biopesticides and did not reduce spore germination or mycelial growth of *B. cinerea* (B03) nor *P. carotovorum* (BA17). In contrast, a proprietary product (PF) reduced mycelial growth of *B. cinerea* (B03) and was further evaluated at doubling concentrations ranging from 0.0625 to 1 ml/L. Mycelial growth of *B. cinerea* and other fungi was completely inhibited by exposure to PF at 1 ml/L. On the other hand, PF was not an effective biopesticides against *P. carotovorum*. PF shows promise for reducing gray mold and will be evaluated as a preharvest spray on tomato plants in the greenhouse.

Key words: Survey, postharvest diseases, tomato, natural product, *Botrytis cinerea*, *Pectobacterium carotovorum*.

INTRODUCTION

Public concern about fungicide residues on raw fruits and vegetables has stimulated research efforts using natural products to reduce incidence of postharvest diseases. Approximately, 25 and 38% of harvested fruits and vegetables, respectively, are lost to postharvest spoilage in the U.S. and global markets (Kantor et al., 1997). Fresh fruit and vegetables can be infected by pathogenic fungi and bacteria during crop growth in the field,

harvesting, postharvest, storage and consumption (Barth et al., 2009). Postharvest diseases cause economic losses in field because of added costs of harvesting, transportation and storage (Adikaram, 1986). The current study focuses on tomato (*Solanum lycopersicum* Mill), which is one of the most important vegetables produced globally, comprising approximately 14% of world vegetable production (Kader, 2004; FAO, 2003). The production value of tomato, estimated at more than $50 billion, makes it the fourth most important commercial crop in the world (Vincent et al., 2013). Tomato is also one of the leading vegetable crops in Oahu, Hawaii. The first objective of this work was to determine the most serious postharvest diseases of tomato in Oahu and to identify the most virulent pathogens associated with each disease. The second objective was to evaluate natural products for biopesticidal activity against pathogenic fungi and bacteria associated with tomato postharvest diseases.

MATERIALS AND METHODS

Survey of postharvest diseases in tomato fruit

A survey was conducted in Oahu, Hawaii extending from November 2014 to April 2015. Samples of infected tomato were randomly collected from 17 locations and 37 markets in Oahu. Two samples (each sample consisting of 10 fruits) were selected from each market. Tissues showing symptoms of postharvest disease were cultured to identify associated pathogens. The percentage of infected tomato based on origin (local or imported) was reported.

Isolation and identification of pathogens

Small (1 cm) sections of infected fruit were cut and surface-sterilized individually in 2% sodium hypochlorite for 1 min and rinsed twice in sterile distilled water. The pieces were dried between sterile Whatman No.1 filter paper and cultured on water agar plates and incubated at 28±2°C for 24 h. Single hyphal tips were transferred to Petri dishes containing V8 medium and incubated at 28±2°C for 5 days under a 12 h photoperiod (Carisse and Van Der Heyden, 2015). Purified cultures were visually identified utilizing laboratory manuals (Dugan, 2006). For bacterial isolates, small sections of rotted tissues were suspended in distilled water for two minutes and the suspension was streaked onto the surface of nutrient agar (NA) plate and plates were incubated at 30°C for 24 h. Basic bacteriological tests including KOH sensitivity, oxidation/fermentation (OF), production of catalase, degradation of sodium polypectate, and hydrolysis of esculin and starch were conducted on each isolated bacteria. All bacterial strains were maintained in freezers (-80°C) until used. Presumptive identifications were confirmed with 16S rDNA sequence analysis (Weisburg et al., 1991).

Pathogenicity tests

Pathogenicity tests were performed on all fungal and bacterial isolates on three types of tomato fruits as previously described by Ahmed et al. (2016). Fruits were selected to be uniform in size and color, free from wounds and showing no symptoms of disease. Fruit were washed with tap water, surface sterilized by dipping in 1% sodium hypochlorite solution for 10 min, rinsed by dipping twice in

sterile distilled water for at least 10 min, and dried in ambient air. A wound (1 mm diameter in 4 mm deep) was made on each fruit using a pipette tip. Mycelial plugs from 10-day-old-cultures of the fungal isolates were inserted into wounds using 0.2 to 10 µl pipette tips. For bacterial stains, fruit were inoculated with 20 µl of a bacterial suspension (1×10^8/CFU). Inoculated fruit were placed in plastic box containing sterile paper towels moistened with sterile water and incubated for 72 h at 23°C. An organism was recorded as pathogenic if symptoms of rot appeared on the tested fruit. The experiments were set up separately for fungal and bacterial isolates with four replications and each experiment was repeated twice.

Virulence tests

Tests were conducted to determine the most virulent isolates on each of the three types of tomato fruit (common market, cherry and grape). Fruits selected were uniform in size and color, free from wounds and showing no symptoms of disease. Virulence of each isolate was determined by measuring the lesion diameter of inoculated fruit after incubation at 23°C for 72 h. The experiments were set up as complete randomized design (CRD) with four replicates. Data were analyzed using SAS 9.2 V.USA and means were compared by Duncan's multiple range tests. Differences at $p < 0.05$ were considered significant. The tests were repeated twice.

Molecular identification

Fungal DNA was extracted from freshly collected mycelium of 10-day-old cultures using the Microbial DNA Isolation Kit (MO BIO, Laboratories, Inc.). The ITS region of the fungal isolates was amplified with the primer pair ITS3 (5-GCA TCG ATG AAG AAC GCA GC-3) and ITS4 (5- TCC TCC GCT TAT TGA TAT GC-3) (Nikolcheva et al., 2003).

Bacterial DNA was extracted from overnight cultures using the Microbial DNA Isolation Kit (MO BIO, Laboratories, Inc.) according to manufacturer's instructions. The 16S rRNA was amplified by PCR for all the isolates using the primers: 16S forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S reverse primer (5'ACGGCTACCTTGTTACGACTT-3'). PCR was performed as previously described by Srinivasa et al. (2012) and Weisburg et al. (1991). Each PCR reaction was run with a negative control (no DNA). The PCR products were electrophoresed on 1.5% agarose gels, stained with 0.4 µg/ml ethidium bromide, and bands visualized with a UV illuminator.

Sequence analysis

Sequence analysis was conducted as described in previous work (Ahmed et al., 2016). PCR product was cleaned utilizing ExoSAP-1T (Affymetrix, Inc., USA). The 5 µl of post-PCR reaction and 2 µl ExoSAP-IT reagents were mixed. The mix was incubated at 37°C for 15 min followed by incubation at 80°C for 15 min. Each purified template was sequenced on both strands using two flanking primers (ITS3- ITS4) for fungal isolates and 16s primers for bacteria. The sequences of ITS 3 and 4 regions, 16s of the tested isolates were edited in order to generate a consensus sequence from forward and reverse sequence in the amplicon using sequence assembly software (DNA BASER). A consensus sequence was analyzed by NCBI BLAST database for fungal and bacterial identities.

Natural controls

Leaf extracts made from *Capsicum annuum* cv. Stocky Red,

Figure 1. Map of Oahu showing sites where fruit samples were collected. Numbers refer to geographic divisions in the island of Oahu. Dots show sites where fruits were collected from separate markets. http://katebraden.com/neighborhoods.shtml.

Capsicum annuum cv. Criolla de cocina, *Capsicum chinense* cv. NuMexsuave, *Tagetes tenuifolia*, *Aloe vera* (leaves and gel), *Origanum vulgare* and *Azadirachta indica* (Neem oil) and a proprietary formulation (PF) (Agrichem, Inc., Australia) were tested. Leaves were extracted by following method described by Wilson et al. (1997) with some modification. Raw leaves were collected in plastic bags and freeze for a minimum of 12 h at 20°C. Plastic bag was removed and leaves fluid were sterilized by using 0.22 µm Millipore filter and stored in 4°C until used.

Assays of antifungal activity

The antifungal activity of nine plant extracts was evaluated against *Alternaria* and *Botrytis* isolates by using an inhibition assay described as the 'poisoned food method' (McCutcheon et al., 1994) with slight modification. A 5 ml sterilized crude extract was mixed with 15 ml of 45°C cooled molten V8 medium and allowed to solidify at room temperature for 30 min. A mycelial disc 6 mm diameter of 7 to 10-day-old cultures was transferred to a Petri plate containing V8 and crude extract. The V8 plate without plant extract served as a control. The antifungal activity of PF was evaluated against *B. cinerea* (B03) at five concentrations 1, 0.5, 0.25, 0.125, 0.0652 ml/L. The most effective PF concentration was reevaluated for the remaining 33 pathogenic fungal isolates. Since different genera have different growth rates on V8 medium, a separate control was established for each fungus by recording the time needed for mycelium to reach the edge of the Petri dish. At that point, the corresponding plate containing PF was measured for inhibition. The experiments were conducted as complete randomized design (CRD) with four replications. Data were analyzed using SAS 9.2

V.USA. Means were compared by Duncan's multiple range tests. Differences at $p < 0.05$ were considered significant. Each experiment was repeated three times.

RESULTS AND DISCUSSION

Survey

Ninety nine fungal and seventeen bacterial isolates were recovered from tomato from the 37 markets in Oahu (Figure 1). Fungal genera were *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Mucor*, *Stemphylium*, *Rhizopus* and *Penicillium*. Bacterial genera were *Acetobacter, Gluconobacter, Klebsiella*, *Leuconostoc* and *Pectobacterium* (Figure 2 and Table 1). Examination of diseased tomatoes based on origin showed 51% were imported from California and Mexico and 49% were grown locally at three sites in Oahu. Some of these pathogens are known to survive on fruit and be spread during transportation, handling and storage (Barkai-Golan, 2001).

Pathogenicity and virulence

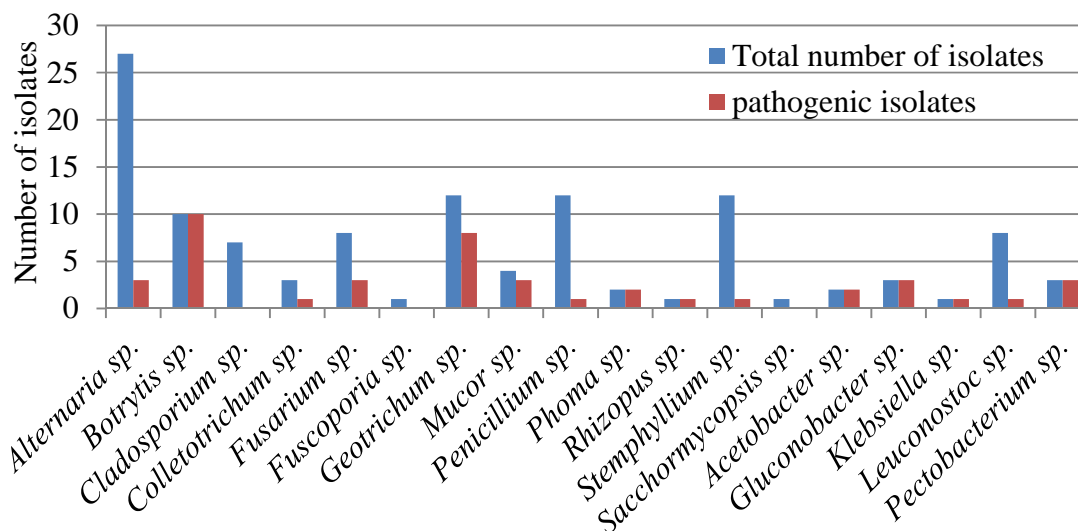The pathogenicity tests showed that 33 of 99 fungal

Figure 2. Genera of fungi and bacteria isolated from infected tomato fruit during a market survey in Oahu.

Table 1. Characteristics of bacterial isolates obtained from infected tomato fruit.

Bacterial stains	Strain No.	Gram[a]	O/F	Pectolytic enzyme production	Esculin hydrolysis	Starch hydrolysis	Catalase production
Gluconobacter frateurii	BA01	-	+/+	-	-	-	+
Klebsiella oxytoca	BA02	-	+/+	-	+	-	+
Leuconostoc sp.	BA03	+	+/-	-	+	-	+
Leuconostoc sp.	BA04	+	+/-	-	+	-	+
Acetobacter sp.	BA05	-	+/+	-	-	-	-
Leuconostoc sp.	BA06	+	+/+	-	-	-	+
Leuconostoc sp.	BA07	+	+/+	-	-	-	+
Leuconostoc sp.	BA08	+	+/-	-	-	-	-
Gluconobacter frateurii	BA09	-	+/+	-	-	-	+
Leuconostoc sp.	BA10	+	+/+	-	+	-	+
Leuconostoc sp.	BA11	+	+/+	-	+	-	+
Pectobacterium carotovorum	BA12	-	+/+	+	+	-	+
Acetobacter sp.	BA13	-	+/+	-	-	-	+
Gluconobacter frateurii	BA14	-	+/+	-	-	-	+
Pectobacterium carotovorum	BA15	-	+/+	+	+	-	+
Leuconostoc citreum	BA16	+	+/+	-	-	-	-
Pectobacterium carotovorum	BA17	-	+/+	+	+	-	+

[a]Presumptive Gram stain determined by formation of sticky stands 30s after adding KOH which is indication of a Gram negative for bacterial cells.

isolates and 10 of 17 bacterial isolates were pathogenic on all three types of tomato fruit (Table 2). Other reports indicate that fungi and bacteria survive and grow saprophytically on tomato (Smilanick, 2004; Agrios, 2005). In this study, the fungal isolates varied in virulence. The pathogenic isolates of *B. cinerea* were highest in lesion diameter range with 16-70, 10-32 and 10-25 mm on three types of tomato fruit common market,

cherry and grape, respectively (Table 3). In addition, *B. cinerea* isolates were varied in their standard deviations, indicating that *Botrytis* isolates are different in virulence level. On the other hand, *B. cinerea* (B03) and *P. carotovorum* (BA17) were significantly more virulent than other isolates when tested on common market, cherry and grape tomato (Tables 4 and 5). Differences in virulence among pathogens are frequently a result of the

Table 2. Pathogenicity tests of fungal and bacterial isolates on artificially wounded fruit of three types of tomato 72 h after incubation at 23°C.

Isolates	Isolate no.	Types of tomato		
		Common market	Cherry	Grape
Alternaria sp.	A01- A03, A05- A19, A21	-	-	-
Alternaria sp.	A04, A20, A22	+	+	+
Botrytis cinerea	B01- B10	+	+	+
Colletotrichum sp.	C01	+	+	+
Colletotrichum sp.	C02	-	-	-
Fusarium sp.	F01- F02, F04, F06	-	-	-
Fusarium sp.	F03, F05,F07- F08	+	+	+
Geotrichum sp.	G01- G03, G05- G11	+	+	+
Geotrichum sp.	G04, G06	-	-	-
Mucor sp.	M01- M02	-	-	-
Mucor sp.	M03,M04	+	+	+
Penicillium sp.	P07	+	+	+
Penicillium sp.	P01-P06	-	-	-
Rhizopus sp.	R01- R03	+	+	+
Stemphylium sp.	S01- S02	+	+	+
Acetobacter sp.	BA5, BA13	+	+	+
Gluconobacter sp.	BA1, BA9, BA14	+	+	+
Klebsiella sp.	BA2	+	+	+
Leuconostoc *sp.*	BA3- BA4, BA6- BA8, BA10- BA11	-	-	-
Leuconostoc *sp.*	BA16	+	+	+
Pectobacterium sp.	BA12, BA15, BA17	+	+	+

+ = rotting; **-** = no rotting

Table 3. The average, range and standard deviation of virulence fungal isolates on common market, cherry and grape tomato 72h after incubation at 23°C.

Fungal genera	Isolates no.	Common market			Cherry			Grape		
			R	SD		R	SD		R	SD
Alternaria sp.	3	29	28-30	1.15	17	13-20	3.49	12	12-13	0.50
Botrytis cinerea	10	48	16-70	19.53	20	10-32	7.03	16	10-25	5.43
Colletotrichum sp.	1	33	-	-	19	-	-	15	-	-
Fusarium sp.	3	35	32-38	2.84	21	20-21	0.76	18	18	0.41
Geotrichum sp.	8	39	35-40	1.87	20	18-25	2.11	20	20	3.29
Mucor sp.	3	56	48-60	6.92	21	20-22	1.07	20	20	0.44
Penicillium sp.	1	35	-	-	21	-	-	12	-	-
Phoma sp.	2	35	31-40	6.12	17	17-17	0.00	11	11-11	0.00
Rhizopus sp.	1	60	-	-	27	-	-	20	-	-
Stemphylium sp.	1	30	-	-	19	-	-	13	-	-

differences in production of cell wall degrading enzymes (CWDEs), oxalic acid and/or secretion of pathogenicity factors (Bellincampi et al., 2014; Kubicek et al., 2014). All *Botrytis* and *Pectobacterium* isolates were pathogenic to the original host from which they were isolated (Figure 2). *B. cinerea* (B03) and *P. carotovorum* (BA17) produced significantly larger lesion diameters (Tables 4 and 5). In previous studies, *Botrytis* and *Pectobacterium* were some

of the most important pathogens causing spoilage decay on tomato (Ahmed et al., 2016; Akbar et al., 2013; Etebu et al., 2013; Fillinger and Elad, 2015).

Molecular identification

A PCR product of 370 bp was amplified efficiently for all

Table 4. Virulence of *Botrytis cinerea* isolates on common market, cherry and grape tomato 72 h after incubation at 23°C.

isolates no.	Lesion diameter (mm)		
	Common market	Cherry	Grape
B01	68.00[a]	27.00[b]	20.00[b]
B02	30.00[c]	22.00[c]	10.00[c]
B03	70.00[a]	32.00[a]	25.00[a]
B04	30.00[c]	12.00[de]	12.00[c]
B05	30.00[c]	16.00[d]	10.00[c]
B06	16.00[d]	14.00[d]	13.00[c]
B07	59.00[b]	22.00[c]	18.00[b]
B08	60.00[b]	22.00[c]	20.00[b]
B09	61.00[b]	25.00b[c]	20.00[b]
B10	59.00[b]	10.00[e]	10.00[c]
Distilled water	01.00[e]	01.00[f]	01.00[d]

*Mean values followed by different letters within a column are different according to Duncan's multiple range tests ($p \leq 0.05$).

Table 5. Virulence of bacterial isolates in common market, cherry and grape tomato 72 h after incubation at 23°C.

Bacterial strains	Strain no.	Lesion diameter		
		Common market	Cherry	Grape
Acetobacter sp.	BA05	27.00[de]	20.00[d]	15.00[c]
Acetobacter sp.	BA13	35.00[c]	23.00[c]	17.00[b]
Gluconobacter sp.	BA01	30.00[d]	21.00[d]	15.00[c]
Gluconobacter sp.	BA09	24.00[e]	14.00[e]	10.00[d]
Gluconobacter sp.	BA14	30.00[d]	14.00[e]	11.00[d]
Klebsiella sp.	BA02	26.00[e]	12.00[f]	11.00[d]
Leuconostoc *sp.*	BA16	37.00[c]	23.00[c]	17.00[b]
Pectobacterium sp.	BA12	47.00[b]	29.00[b]	24.00[a]
Pectobacterium sp.	BA15	35.00[c]	23.00[c]	17.00[b]
Pectobacterium sp.	BA17	66.00[a]	31.00[a]	24.00[a]
Distilled water	control	01.00[f]	01.00[g]	01.00[e]

*Mean values followed by different letters within a column are significantly different according to Duncan's multiple range tests ($p \leq 0.05$).

fungal isolates. The ITS3-ITS4 region of identified fungi at > 98% similarity was compared with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *B. cinerea* was counterpart at 100% similarity. A PCR product with expected size 1400 bp was amplified for all bacterial isolates and NCBI BLAST of the 16S region identified bacteria at 99 to 100%. *Pectobacterium* isolates matched 100% with *P. carotovorum*.

Inhibitory effect of plant extracts on fungal colony

The crude leaf extracts of *C. annuum* cv. 'StockyRed', *C. annuum* cv. 'Criolla de cocina', *C. chinense* cv. 'NuMexsuave', *T. tenuifolia*, *A. vera*, *O. vulgare* and *A. indica* Neem oil showed no measurable inhibition of mycelial growth for *Alternaria* sp. or *Botrytis* sp. In contrast, PF completely inhibited mycelial growth of both fungi (Figure 3). The most effective PF concentration was 1 mL/L that completely inhibited growth of all 33 of the other tested pathogenic fungi (Table 6).

Conclusion

B. cinerea and *P. carotovorum* were the most virulent postharvest pathogens of tomato in Oahu. Thirty percent of the fungal and 58% of the bacterial isolates were pathogenic. A natural proprietary product (PF) had sufficient antifungal activity to completely inhibit mycelial
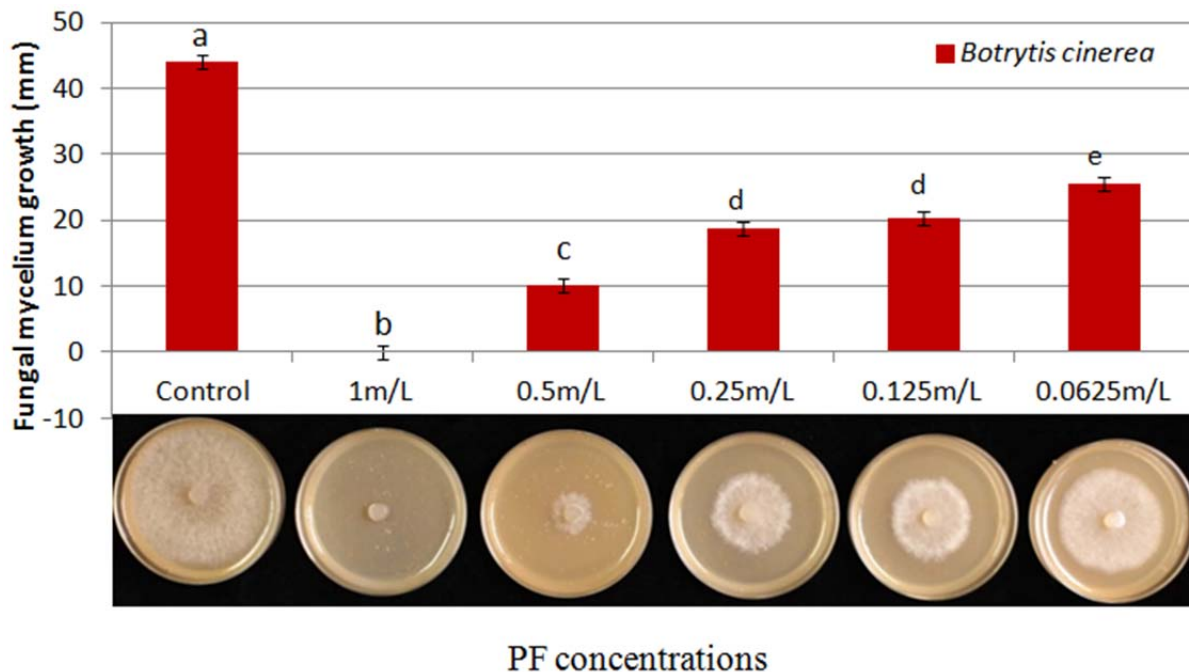
Figure 3. Mycelial growth of *Botrytis cinerea* (B03) on agar containing PF at five different concentrations.

Table 6. Effect of PF (1 ml/L) on mycelial growth of 33 pathogenic fungi 72 h after incubation at 23°C using an inhibition assay.

Fungal isolates	Isolates no.	Colony diameter (mm)*
Control (V8 only)	---	44.00^a
Alternaria sp.	A04	00.00^e
Alternaria sp.	A20	00.00^e
Alternaria sp.	A22	00.00^e
Botrytis sp.	B01	03.00^c
Botrytis sp.	B02	00.00^e
Botrytis sp.	B03	00.00^e
Botrytis sp.	B04	00.00^e
Botrytis sp.	B05	04.00^b
Botrytis sp.	B06	00.00^e
Botrytis sp.	B07	04.00^b
Botrytis sp.	B08	00.00^e
Botrytis sp.	B09	00.00^e
Botrytis sp.	B10	00.00^e
Colletotrichum sp.	Col1	00.00^e
Fusarium sp.	F03	00.00^e
Fusarium sp.	F07	02.00^d
Fusarium sp.	F08	00.00^e
Geotrichum sp.	G01	00.00^e
Geotrichum sp.	G02	00.00^e
Geotrichum sp.	G03	00.00^e
Geotrichum sp.	G05	00.00^e
Geotrichum sp.	G06	00.00^e
Geotrichum sp.	G07	00.00^e
Geotrichum sp.	G08	00.00^e

Table 6. Contd.

Geotrichum sp.	G09	00.00^e
Mucor sp.	M01	00.00^e
Mucor sp.	M03	00.00^e
Mucor sp.	M04	00.00^e
Penicillium sp.	P07	00.00^e
Phoma sp.	Ph01	00.00^e
Phoma sp.	Ph02	00.00^e
Rhizopus sp.	R01	03.00^c
Stemphyllium sp.	S01	00.00^e

*Mean values followed by different letters within a column are significantly different according to Duncan's multiple range tests ($p \leq 0.05$).

growth of all isolated fungi but had no effect on the bacteria. This natural product is a potential alternative to synthetic fungicides in reducing postharvest gray mold disease.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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