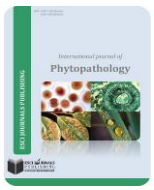

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CHARACTERIZATION AND CONTROL OF *PESTALOTIOPSIS* SPP. THE CAUSAL FUNGUS OF GUAVA SCABBY CANKER IN EL-BEHEIRA GOVERNORATE, EGYPT

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A B S T R A C T

During 2013-2014 growing seasons, forty three isolates of *Pestalotiopsis* spp. were recovered from guava leaves and fruits showed scab symptoms from different regions in EL-Beheira governorate. Five *Pestalotiopsis* species were recognized according to the morphological characteristics of fungal colony (Colony color, Size and number of acervulii) and conidia (length, width, and color of median cells, length and the number of apical and basal appendages); they were *P. psidii*, *P. microspora*, *P. clavispora*, *P. neglecta* and *Pestalotiosis* spp. All the isolates recovered were pathogenic to the cv. Balady of guava fruits. However, *P.psidii* isolates were the most highly pathogenic followed by *P. neglecta*, *P. clavispora*, *P. microspora* and *Pestalotiopsis* spp, respectively. RAPD-PCR analysis using five random oligonucleotide primers revealed DNA fingerprints and considerable variations were revealed with primers tested. Bar primer showed a common band for all *Pestalotiopsis* isolates and species at 500bp, while BAQ, 18 and A9B4 exhibited banding pattern similar for all isolates of the same species which were different from that of the other species. Scab disease control of infected fruits by chitosan as a natural product was tested. The *in vitro* 2.5% chitosan application significantly inhibited the growth of *Pestalotiopsis* spp. tested by 86.53% on agar plates. The *in vivo* tests on fruits, the chitosan treatment to artificially infected fruits reduced the development of symptoms at the different chitosan concentrations, *i.e.,* 1.5%, 2% and 2.5%. The 2.5% chitosan was the most effective concentration for scab disease control in guava fruits. It is the first report of identification five different *Pestalotiopsis* species affecting guava fruits and leaves in EL-Beheira Governorate, Egypt. Also, the study supported the view that chitosan offers a safe alternative to synthetic fungicides in postharvest scabby control and could be considered as a potential agrochemical of low environment impact.

Keywords: Guava- scabby canker- *Pestalotiopsis* spp.- *Pestalotiopsis psidi*- Chitosan- RAPD PCR.

INTRODUCTION

Guava (*Psidium guajava* L.) is grown in nearly every tropical and sub-tropical country in the world. Guava is very important fruit having 82% water, 0.7% protein, 11% carbohydrates and enough amounts of vitamins A, B, B2 and C plus some minerals (Bardi, 1975). However, diseases play a crucial role in limiting the yield of guava production. Scabby fruit canker, caused by *Pestalotiopsis* spp., is one of the most common fruit diseases in guava-growing areas and affects all developmental stages of guava fruit (Kwee & Chong, 1990). The genus *Pesatlotiopsis* was early descried by (Nag Rag, 1993). He cleared that the conidiomata of the genus was variable and ranging from acervuli to pycnidia. Conidiomata can be immersed to erumpent, unilocular to irregularly plurilocular with the locales occasionally incompletely divided and dehiscence by irregular splitting of the apical wall or overlaying host tissue. Conidiophores partly or entirely develop inside the conidiomata, and they can be reduced to conidiogenesis cells which are discrete or integrated, cylindrical, smooth, colorless and invested in mucus.

Scabby canker can drastically reduce fruit yield during the pre-harvest stage, and can also, lead to fruit losses during postharvest storage (Kaushik *et al*., 1972 and Kwee & Chong, 1990). *Pestalotiopsis* species are usually found in tropical and temperate ecosystems (Jeewon *et al*., 2004; Tejesvi *et al*., 2007 & 2009; Ding *et al*., 2009 and Liu *et al*., 2008 & 2009), and many cause plant

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disease in a variety of plants including canker lesions, shoot dieback, leaf spots, needle blight, tip blight, grey blight, scabby canker, severe chlorosis, fruit rots and leaf spots (Trapero *et al*., 2003 ; Sousa *et al*., 2004 and Espinoza *et al*., 2008). The genus *Pestalotiopsis* Steyaert is a heterogenous group of coelomycetous fungi consisting of 230 described species (Tejesvi *et al*., 2009) that are differentiated primarily on conidial characteristics such as size, septation, pigmentation, and presence or absence of appendages (Nag Rag 1993 and Sutton 1980). Some species have also, been identified based on their host occurrence (Kohlmeyer & Volkmonn-Kohlmeyer, 2001 and Chen *et al*., 2002). In the recent years, precise assessment of diversity and identification of fungi had a great impact on fungal taxonomy due to rapid developments in molecular techniques (Phillips *et al*., 2007; Zhu *et al*., 2008 and Thongkantha *et al*., 2009). Fungal identification is more reliable when classical and molecular approaches are combined (Hyde & Soytong, 2008 and Than *et al*., 2008). Despite the broad application of random amplified polymorphic DNA (RAPD) based genetic markers for analysis of genetic diversity of fungal endophytes, little information is available on the species diversity of endophytes. RAPD analysis have been successfully used to identify strains (Pryor & Gilbertson, 2000 and Jana *et al*., 2003), characterize races (Malvick & Grau, 2001) and to analyzes virulence variability related to genetic polymorphisms (Kolmer & Liu, 2000 and Eman El-Argawy, 2012) in phytopathogenic fungi. RAPD can also, be used to detect genetic diversity in species of *Pestalotiopsis* (Tejesvi *et al*., 2007). At present at least 23 *Pestalotiopsis* species have been reported as endophytes some of which produce secondary metabolites with a great potential for anti-microbial to the control of plant diseases and anti-tumor medicinal application (Wei & Xu, 2004 ; Wei *et al*., 2005; Ding *et al*., 2009 ; Liu *et al*., 2009 ; Aly *et al*., 2010 and Xu *et al*., 2010).

Traditionally, the use of synthetic fungicides has been the preferred post-harvest treatment to control this microorganism (Aked *et al*., 2001). However, over time the reported use of fungicides has resulted in serious problems; the pathogens have developed resistance and residue levels have considerably increased (Mari *et al*., 2003). Chitosan is a naturally occurring polysaccharide derived from chitin that has exhibited potential to control several post-harvest plant diseases and to extend the shelf life of fruits and vegetables (Meng *et al*., 2008; Badawy & Rabea, 2009 and Eman El-Argawy, 2012). Several reports have shown that chitosan has antimicrobial activity and can interfere with spore germination and mycelial growth of phytopathogenic fungi (Rebea *et al*., 2003 and Muňoz *et al*., 2009). It was reported that chitosan confers protection against *Botrytis cinerea* in *Vitis vinifera* and controlled grey mould in cucmber plants (Romanazzi *et al*., 2006 and Nascimento *et al*., 2007). Tomato seeds were also, protected against *Fusarium oxysporum* after immersion into a chitosan solution (Borges *et al*., 2000).

The objectives of the present study were to: 1) identify the causal agent of scab canker affecting guava fruits collected in EL-Beheira governurate, 2) reveal its morphological characteristics, genetic variability and pathogenicity on guava fruits and 3) to investigate the potential of chitosan treatment to control scab of guava fruits.

MATERIALS AND METHODS

Isolation and identification of the causal fungus: Naturally infected samples from fruits (52 fruit samples) and leaves (23 samples) of guava cultivar (cv. Balady) fruits and leaves samples of guava (cv. Balady) were collected randomly from different orchards in EL-Beheira governorate (*i.e.,* Abo-Homos, Edkou, EL-Nubaria and EL-Mahmudia). Samples were washed thoroughly in running tap water for 5 minutes, surface disinfected with 70% (v/v) ethanol 1min and 1% (v/v) NaOCl (1min), followed by rinsing with sterile water and allowed to surface dry under sterile conditions. Then, samples were cut into small pieces and plated on potato dextrose agar medium supplemented with streptomycin (100 mg/L). The plates were wrapped and incubated at 25±2ºC for 3 days in the dark. For the purification of the isolates hyphal tips of emerging fungal colonies were transferred to agar plates. The purified cultures were stored in PDA slants at 4°C and sub-cultured every three months.

Morphological characterization: The recovered fungal isolates were grown on PDA and incubated at 25±2°C in continuous light, and culture morphology was examined after 7 days. Colony color was defined according to Raynor (1970). Spore size was determined by measuring the length and width of 30 to 40 arbitrarily selected conidia from a conidial suspension of each isolate that was prepared in SDW (sterile distilled water). The isolates were identified initially by their morphological and cultural characteristics (*i.e*.,

size of conidia, color and length of median cells, thickness and length of a pical appendages, to those described in Guba's monograph of *Monochaeita* and *Pestalotia* (Guba, 1961), Strobel *et al*. (1996), Keith *et al.* (2006), Das Ranjana *et al*. 2010 and Watanabe *et al*. (2010) and Maharachchikumbura *et al.* (2011).

Pathogenicity tests: Pathogenicity tests were performed according to Keith *et al*. (2006). Direct inoculation technique on Mature guava fruits freshly removed from the tree was conducted. Before inoculation, fruits were surface disinfected by immersion in 10% bleach solution (0.5% Sodium hypochlorite) for 2 min, then rinsed in SDW, and air-dried in a laminar flow hood. Fruits were placed in plastic chambers containing moistened paper towels. Fruits (Psidium guajava cv. Balady) were wounded with a sterile cork borer and inoculated with actively growing mycelial discs (3mm diameter) taken from 5-7 day old culture of fungal isolates. Inoculated samples were incubated at room temperature (25±2°C) and the length of necrotic lesions obtained was determined 5 days after inoculation. Controls were inoculated with PDA discs only. Three replicates were made for each isolate. To fulfill Koch's postulates, diseased tissues were placed on PDA and observed for colonies typical of the pathogen Keith *et al*. (2006).

Molecular characterization: A total number of ten isolates belongs to five different *Pestalotiopsis* spp. were randomly chosen based on their reaction on artificially inoculated guava fruits and tested for the DNA banding pattern. These isolates were PS3, PS9, PS19 from *P. psidii*, PS23 from *Pestaotiopsis* spp, PS27, PS31 from *P. microspora*, PS33, PS34 from *P. neglecta* and PS40, PS42 from *P. clavispora.*

Genomic DNA extraction: Isolates were grown on potato dextrose broth for 10 days at 25 ± 2ºC in darkness. Mycelial mats were harvested by filtration using filter paper No. 1. Then, DNA was extracted using the hexadecyltrimethyl ammonium bromide method according to Murray and Thompson (1980). Concentration and purity of the obtained DNA were determined and adjusted using the standard methodology of Sambrook *et al*. (1989).

Random amplified polymorphic DNA (RAPD): DNA from *Pestalotiopsis* spp. isolates was amplified by the RAPD methods (Williams *et al*., 1990) using five random oligonucleotide primers shown in (Table 1). Amplification was conducted in a Thermocycler (Eppendorf, Germany) programmed for 35 cycles. The entire reaction mixtures were loaded on 1.5% agarose gel and amplified DNA fragments were resolved by electrophoresis , stained by ethidium bromide and photographed under UV light (320 nm) according to Jana *et al*. (2003). All chemicals were Bio-Rad products.

Phylogenetic analysis: Band patterns of DNA developed in the RAPD-PCR analysis were scored visually for each tested isolate. Dendrogram of the phylogenetic relationship was produced using the software program "Statistica version 5.0" according to Rholf (2000).

Table 1. Nucleotide sequences of 5 primers used to screen the polymorphism of *Pestalotiopsis* spp. isolates recovered in the present study.

Primer code	Nucleotide sequence (5′ to 3′)
BAR	CCA GGC AAT TTC ATC AAG CC
BAQ	GGT CTT GAA GTC GAG CGC AG
18	CGC ATA GGA CCC GAT GCG AG
A9B4	GGT GAC GCA GGG GTA ACG CC
A9B10	GGA CTG GAG GTG GAT CGC AG

Effects of chitosan on fungal growth and disease reduction

The *in vitro* effect of different concentrations of chitosan on the mycelium growth of the recovered *Pestalotiopsis* spp. isolates: The chitosan solution was prepared by dissolving chitosan 2.5 % deacetylated in 0.25 N HCl with continuous stirring at 50 ºC. Insoluble material, were removed by centrifugation and chitosan was precipitated by neutralization with 1 N NaOH, washed three times with deionized water and air dried (El Ghaoth *et al*., 1991) . For incorporation into the PDA, purified chitosan was dissolved by stirring in 0.25 N HCl and adjusting the pH to 5.6 using 1 N NaOH. Chitosan solution was added to the PDA medium to obtain final concentrations of 0, 1.5, 2 and 2.5 % after autoclaving before pouring into Petri dishes. A 5 mm diameter disc from the margin of an actively growing PDA culture of the tested isolate was placed upside down at the center of each Petri dish. Four replicates were used for each chitosan concentration. The inoculated plates were incubated at 25 ºC for 5 days in the dark. Mycelial growth was determined by measuring colony diameter. Five isolates exhibited the highest aggressiveness in the pathogenicity test for each Pestalotiopsis species tested were used in the control studies and the same isolates were inoculated and incubated under the same condition but without addition of chitosan to the agar plates and served as control.

Effect of chitosan to control guava scabby canker disease: Based on Jinasena *et al*. (2011) with some minor modifications. Freshly harvested mature fruits of guava (cv. Balady) were selected on the basis of uniformity of size and absence of any visible symptoms. Fruits were surface sterilized in 10 % NaOCl for 5 min, rinsed in sterile water and then air dried. Superficial wounds in the epidermis, made by a sterile scalpel (0.5 – 1.0 cm deep), were treated with 15 µl of the chitosan concentrations (1.5, 2 & 2.5 %). For inoculation with the fungus, a 4 mm diameter disc of PDA was removed from the margin of an actively growing PDA culture and placed mycelium side - up on the wound. Replications were made using every concentration (four each) on inoculated fruits and placed in four separate plastic chambers (one group of replication in each). Moistened paper towels were placed in the plastic chambers containing the inoculated fruits and incubated at 25 ºC. Lesion diameters on the treated fruits were measured every day up to 5 days after inoculation.

Statistical analysis: The obtained data were statistically analyzed using the American SAS/STAT Software, version 6 and means were compared by the least significant difference test (LSD). (SAS Institute, 2000).

EXPERIMENTAL RESULTS

Occurrence and frequency of the scab of guava and the causal fungus in EL-Beheira governorate: In the 2013-2014 growing seasons, a survey was conducted in four different regions for guava scab in EL-Beheira governorate and a number of (52 fruit samples) and leaves (23 samples) of guava cultivar (cv. Balady) showing disease symptoms were collected based on symptoms appeared on fruits or leaves. Forty three Pestalotiopsis spp. isolates were recovered from leaves and fruits showed scab symptoms and five Pestalotiopsis species were identified; they were P. psidii, P. microspora, P. clavispora, P. neglecta and Pestalotiopsis spp. (Figure 1).

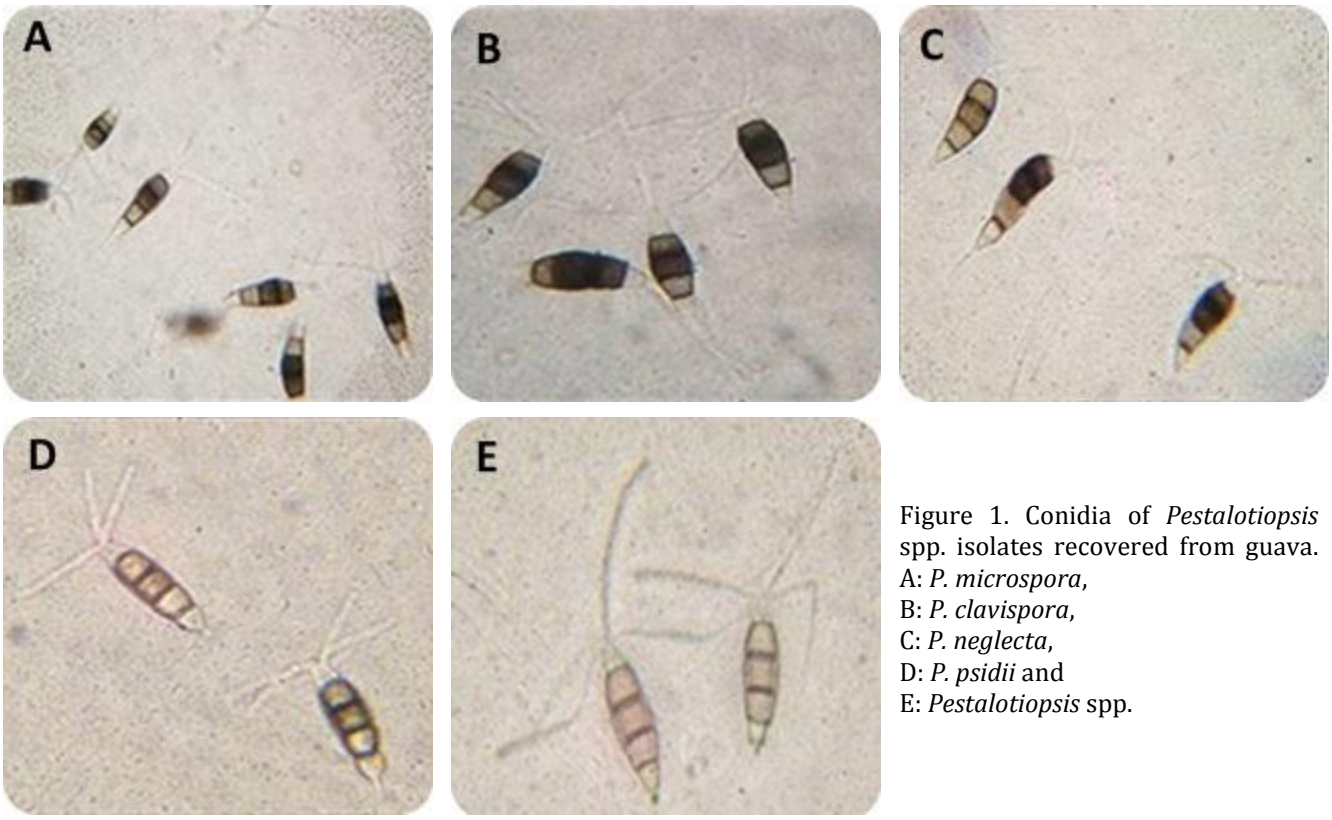

Figure 1. Conidia of *Pestalotiopsis* spp. isolates recovered from guava.
A: *P. microspora*,
B: *P. clavispora*,
C: *P. neglecta*,
D: *P. psidii* and
E: *Pestalotiopsis* spp.

Among all the four surveyed regions, Abo-Humos recorded the highest number of *Pestalotiopsis* spp. (19 isolates) while the lowest number of isolates (4) recovered was from EL-Mahmodya. Meanwhile, both Edkuo and EL-Nubaria yielded 14 and 6 isolates respectively (Table 2). *P. psidii* was most prevalent and recorded in three regions, *i.e.,* Abo-Humos , Edkuo and EL-Mahmudya out of the four surveyed regions with 11, 6 and 4 isolates respectively and none of its isolates were recovered from EL-Nubaria. *Pestalotiopsis* spp.

was only found in EL-Nubaria with two isolates and *P. neglecta* was also only found in Edkuo and scored four isolates. On the other side, both *P. microspora* and *P. clavispora*, were recovered from also three out of the four surveyed regions, *i.e.,* EL-Nubaria, Edkou, and Abo-Humos and none of their isolates were recovered from EL-Mahmudya (Table 2 & Figure 2). The recovered isolates were more frequently isolated from diseased fruits showed scab symptoms comparing to that recovered from leaves. *P. psidii, P. microspora* and *P. clavispora* species were recovered from both diseased fruits and leaves while the other species, *i.e., P. neglecta* and *Pestalotiopsis* spp. were only recovered from the infected fruits (Table 2 & Figure 3).

Table 2. Number of *Pestalotiopsis* spp. recovered from fruits and leaves of guava samples collected from different regions in EL-Beheira governorate during 2013-2014 growing seasons.

Pestalotiopsis spp./ Reigon		*P.psidii*	*P.microspora*	*P.neglecta*	*P.clavispora*	*Pestalotiopsis spp.*	Total
EL-Mahmudya	F	4	-	-	-	-	4
	L	-	-	-	-	-	-
	T	4	-	-	-	-	4
EL-Nubaria	F	-	1	-	-	2	3
	L	-	1	-	2	-	3
	T	-	2	-	2	2	6
Edkou	F	4	2	4	-	-	10
	L	2	-	-	2	-	4
	T	6	2	4	2	-	14
Abo-Humos	F	5	4	-	-	-	9
	L	6	1	-	3	-	10
	T	11	5	-	3	-	19
Total	F	13	7	4	-	2	26
	L	8	2	-	7	-	17
	T	21	9	4	7	2	43

F: Fruits; L: Leaves; T: Total: No isolates was recovered

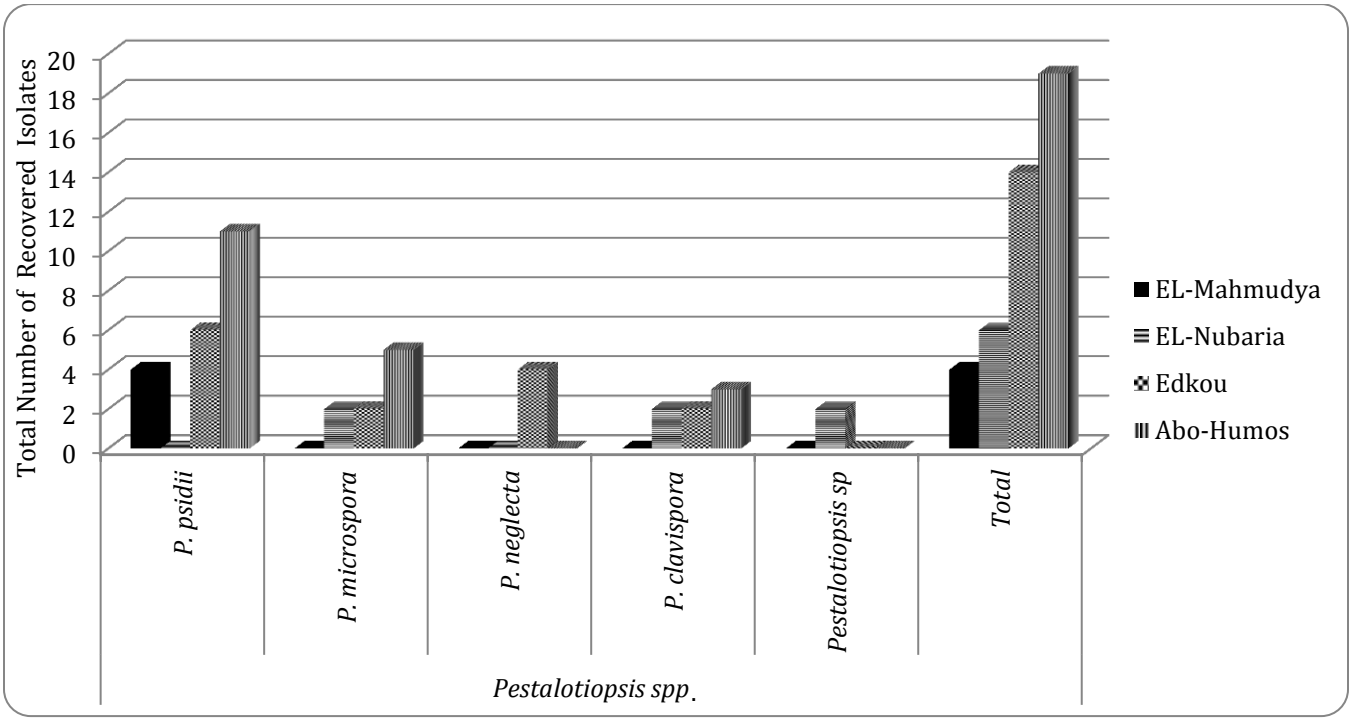

Figure 2. Number of *Pestalotiopsis* spp. recovered from guava fruit and leaves samples showed scab symptom collected from in EL- Beheira governorate during 2013-2014 growing seasons.

Characteristic of the recovered *Pestalotiopsis* isolates

Colony phenotypes: Colonies of the recovered isolates were cottony and becoming darker as fungi aged on PDA medium. Black acervuli were formed superficially or submerged scattered on the PDA medium. The colony characteristics of *P. psidii* isolates were mostly creamy and none of its recovered isolates was of the white colony phenotype. On the contrary *P. neglecta* colonies were all of the white colony phenotype. However, the two morphology phenotypes were occurred in *P. microspora* and *P. neglecta* in almost equal frequencies (Table 3, Figure 4 & 5).

The back view of the recovered isolates of the *P. psidii* isolates was almost of yellow to light yellow appearance while the analyzed *P. microspora* colonies were of orange to light orange appearance. The *P. clavispora* colonies, however, were of yellowish brown or of saffron appearance while colonies of *P. neglecta* (3 isolates) were mostly were of light orange appearance for its back view (Table 3, Figure 4 & 5). Meanwhile, *P. psidii*, *P. neglecta* and *P. clavispora* showed big acervulii and none of the recovered isolates for these species produced any small acervulii. However, the small acervulii were only found in *P. microspora* and *Pestalotiopsis* spp.

Table 3. Colony phenotypes in cultures of *Pestalotiopsis* spp. isolates recovered from naturally infected guava fruits and leaves*.

Characteristics	Colony color								Acervuli	
	Front view			Back view						
Pestalotiopsis spp.	Creamy	White	Yellow	L-yellow	Orange	L-orange	Yellowish brown	Saffron	Big	Small
P. psidii	21	0	11	10	0	0	0	0	21	0
P. microspora	5	4	0	0	5	4	0	0	0	9
P. neglecta	0	4	1	0	0	3	0	0	4	0
P. clavispora	4	3	0	0	0	0	3	4	7	0
Pestalotiopsis sp	0	2	0	0	2	0	0	0	0	2
Total	30	13	12	10	7	7	3	4	32	11

* Isolates were grown on PDA with continuous light for seven days on 25±2ºC. 0 =No isolates recorded.

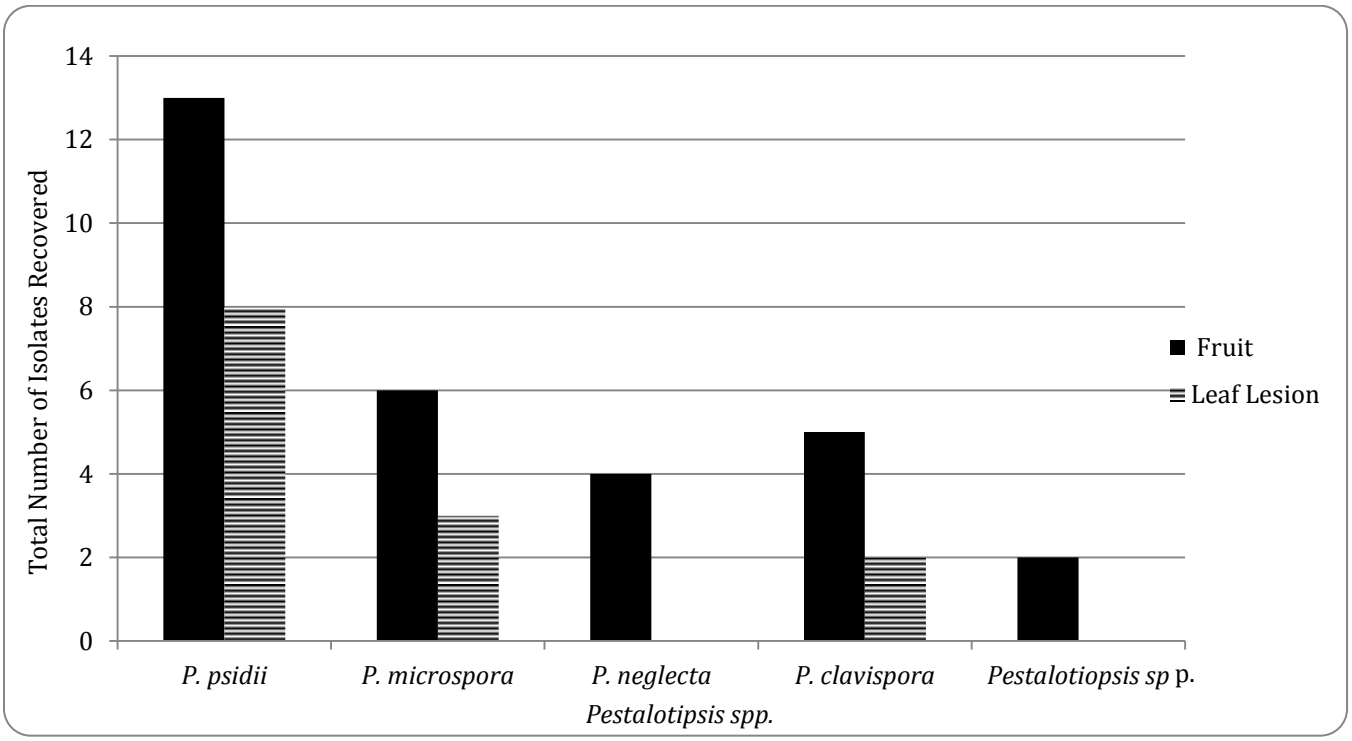

Figure 3. Number of Pestalotiopsis isolates recovered from naturally infected guava fruits and leaves.

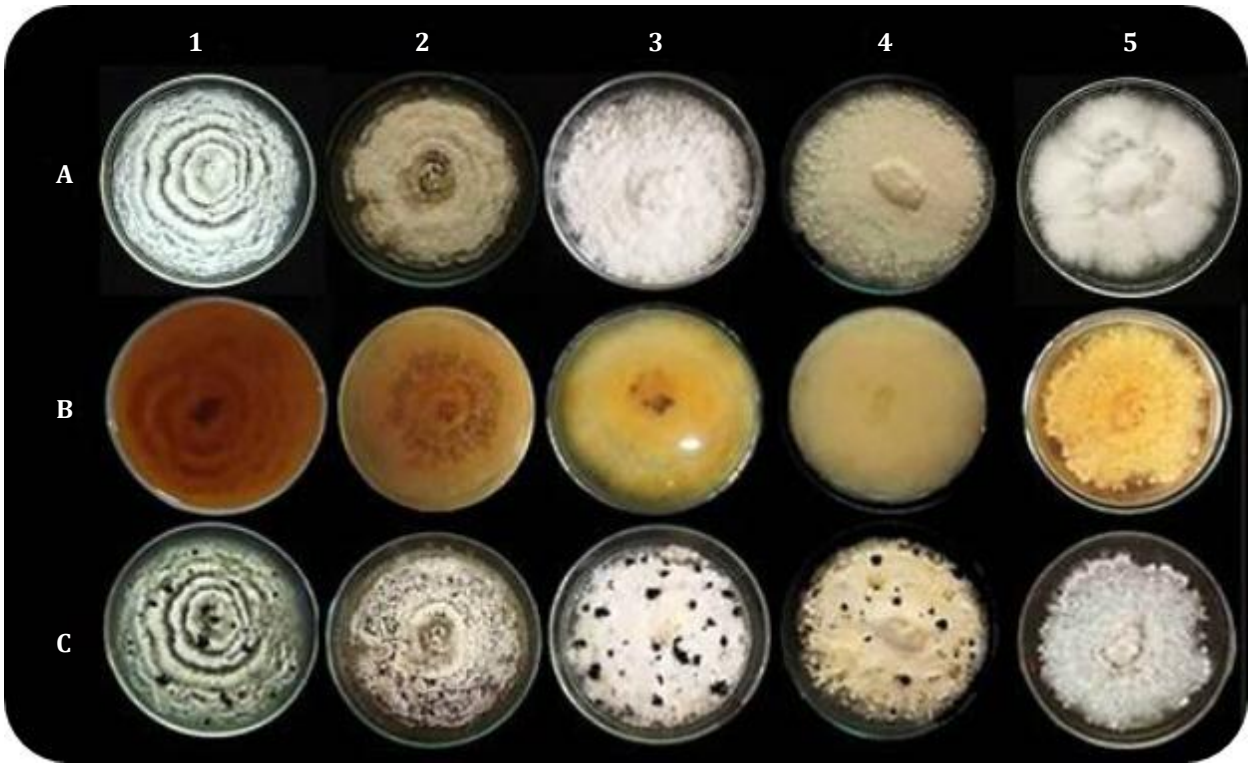

Figure 4. Colony phenotypes of *Pestalotiopsis* spp. on PDA, 7 - 10 days after incubation at 26ºC. 1= *P. clavispora*, 2= *P. microspora*, 3= *P. neglecta*, 4= *P. psidii* and 5= *Pestalotiopsis* spp. A= Front view, B= Back view, C= Acervulii.

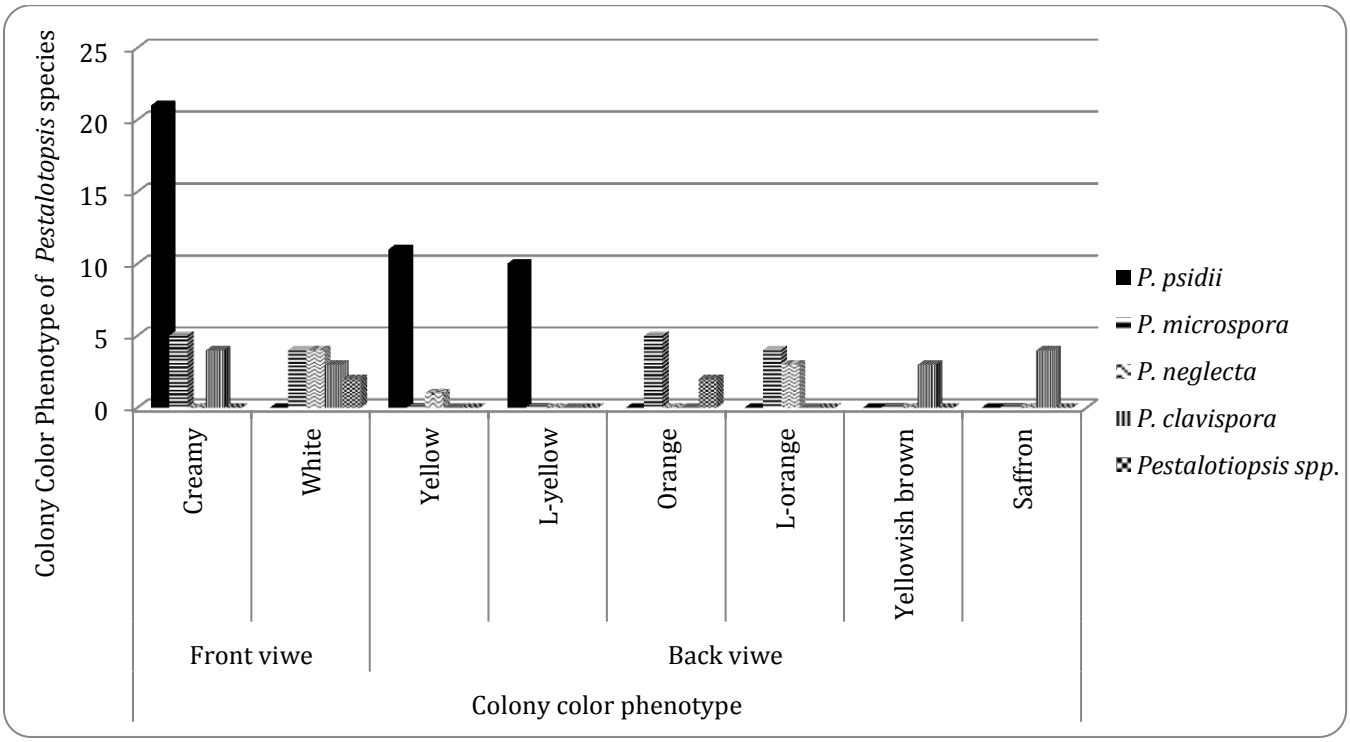

Figure 5. Colony color phenotypes of *Pestalotiopsis* spp. isolates recovered in the survey. i.e., *P. psidii, P. microspora, P. neglecta, P. clavispora* and *Pestalotiopsis* spp. Phenotypes were recorded on PDA 7 - 10 days after incubation at 26ºC.

Conidia phenotypes of the recovered *Pestalotiopsis* species: Conidia of *P. psidii* isolates showed 23.09±0.3 µm in length and 4.8±0.4µm in width. Three apical appendages were 20.48±0.3 µm in length, while the basal

appendages were small hyaline pedicel, of 3.79±0.3µm in length. The median cells were pale brown (Table 4).

Conidia of *P. microspora* isolates were of 25.21±0.3 µm in length and 5.63±0.3 µm in width. Two to three apical appendages and one basal appendage were always observed. Apical appendages varied from 18.04±0.4 µm in length, while the basal appendage was 4.06±0.2 µm in length. Median cells were found to be brown (Table 4).

Conidia of *P. neglecta* isolates were of 25.77±0.3 µm in length and 6.7±0.2 µm in width. Three to four apical (mostly three) appendages with a rounded apical end and one basal appendage were always observed. The apical appendages were 30.55±0.3 µm in length, while the basal appendage were 4.62±0.2 µm in length. The median cells were brown (Table 4).

Conidia of *P. calvispora* isolates were of 24.82±0.5 µm in length and 7.74±0.2 µm in width. Two to four (Three being the most frequently observed) apical appendages and one appendage basal were always observed. Apical appendages were 28.44±0.3 µm in length, while the basal appendage were 7.16±0.3 µm in length. The dark brown color was the main color of the observed median cells (Table 4).

Table 4. Characteristics of conidia of *Pestalotiopsis* spp. isolates recovered from guava samples during 2013-14 seasons.

Number of apical appendages (range)	Size of appendages(µm)		Conidia		Color of median cells	*Pestalotiopsis* spp.
	Basal	Apical	Width (µm)	Length (µm)		
2-3	3.79±1.3	20.48±1.3	4.8± 1.4	23.9±1.3	Pale brown	*P. psidii*
2-3	4.06± 1.2	18.04± 1.5	5.63± 1.3	25.21±1.3	Brown	*P. microspora*
3-4	4.62± 1.3	20.55±1.3	6.7±1.2	25.77±1.3	Brown	*P. neglecta*
2-4	7.16±1.3	28.44±1.3	7.74±1.2	24.82±1.5	Dark brown	*P. clavispora*
3-4	0.55± 1.2	30.05±1.4	7.55±1.2	25.15±1.4	Pale olivaceous	*Pestalotiopsis* spp.

*Fungal isolates were grown on PDA with continuous light for seven days on 25±2ºC.

Conidia of *Pestalotiopsis* spp. isolates were of 25.15±0.4 µm in length and 7.55±0.2 µm in width. Three to four apical (usually four) appendages and one basal appendage were always observed .The apical appendages were 30.05±0.4 µm in length , while the basal appendage was very short, 0.55±0.2 µm in length. The median cells were unique with pale olivaceous color (Table 4).

Pathogenicity: All of the 43 recovered isolates belonging to the five species *P. psidii, P. microspora, P. neglecta, P. clavispora* and *Pestalotiopsis* spp. proved to be pathogenic to guava fruits cv, Balady to different degrees (Figure 6). The *P. psidii* isolates were the highly pathogenic and exhibited mean lesion diameter of 3.64 cm. No significant differences were revealed between *P. neglecta* and *P. clavispora* isolates recovered and they exhibited mean lesion diameters of 2.62 cm and 2.60 cm respectively. On the other hand *P. microspora* isolates showed intermediate mean lesion diameter of 2.33 cm. the *Pestalotiopsis* spp. isolates, however, were the most weakly pathogenic and scored the lowest mean lesion diameter of 1.82 cm.

Molecular characteristics of the recovered *Pestalotiopsis* isolates

Random amplified polymorphic DNA (RAPD): The use of five primers in RAPD-PCR showed considerable variation among the ten tested isolates of *Pestalotiopsis* isolates and the related species on basis of the amplified product band patterns revealed with each primer (Figure 7). Most primers tested succeeded to reveal polymorphic patterns among different *Pestalotiopsis* species. The Bar primer showed a common band for all *Pestalotiopsis* isolates and species at 500 bp, while BAQ, 18 and A9B4 exhibited similar banding patterns for the isolates of the same species which were different from that of the other species. The A9B10 primer, however, did not reveal obvious variation among the analyzed isolates. Meantime, analysis of the obtained banding pattern using Nei & Lis coefficient revealed high similarity (89%) between PS27 isolate and PS31 isolate of *P. microspora* while between PS23 from *Pestalotiopsis* spp. and PS42 from *P. clavispora* the similarity coefficient was as low as 55%.The low similarity coefficient could indicate different species of *Pestalotiopsis* spp. (Table 5) as the high similarity was observed between the same species. This was obvious in the case of PS3, PS9 and PS19 isolates belong to the *P. psidii* where the similarity matrix between PS3 and PS9 was 88%, PS3 and PS19 80% and 86% between PS9 and PS19. On the same trend this were found between PS40 and PS42 of *P. clavispora* with 78%, PS33 and PS34 belonged to *P. neglecta* with 85%, and 89% for similarity coefficient between PS27 and PS31 from *P. microspora*.

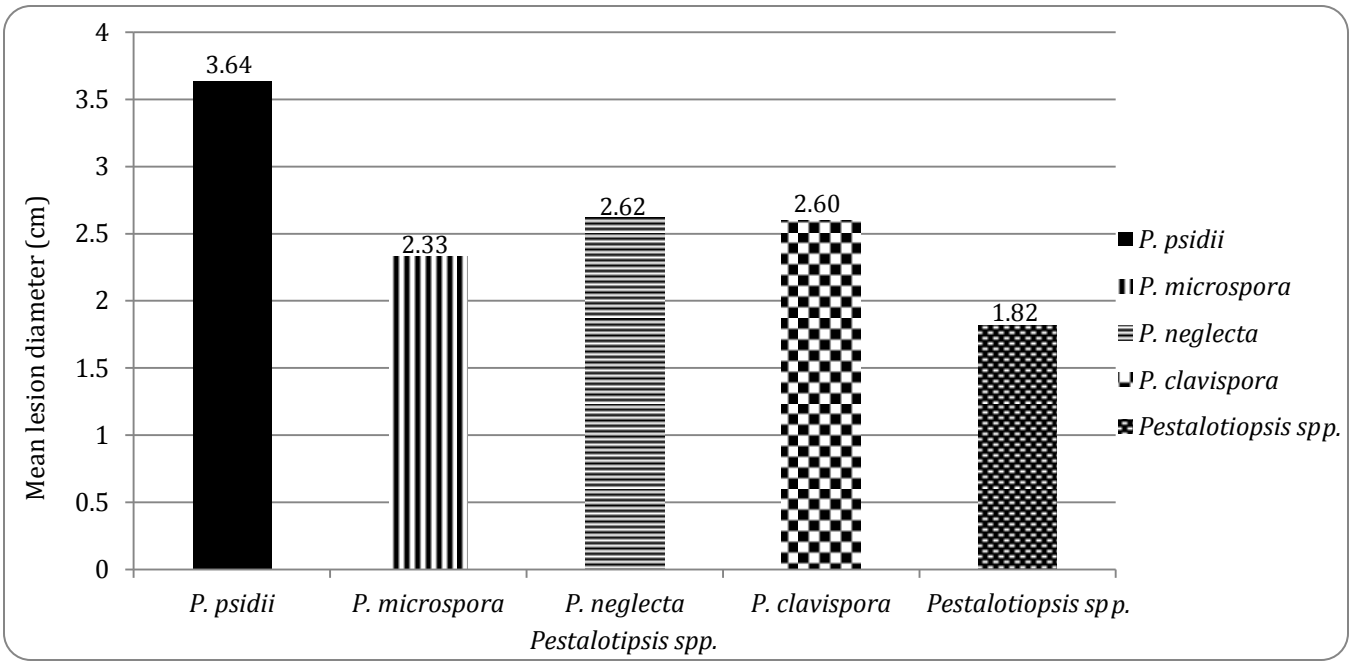

Figure 6. Mean diameter lesions, incited by *Pestalotiopsis* spp. isolates recovered in the survey, on guava fruits (cv. Balady) in the pathogenicity test.

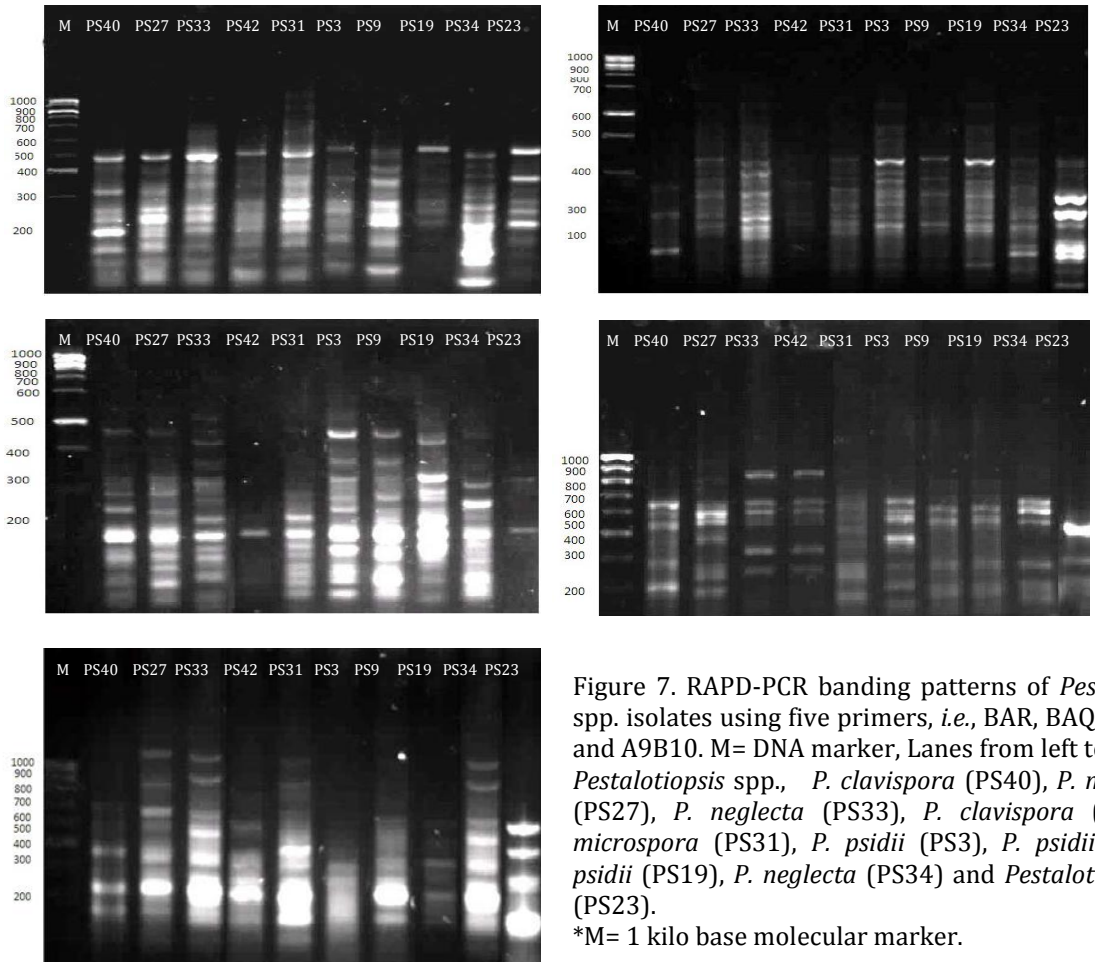

Figure 7. RAPD-PCR banding patterns of *Pestalotiopsis* spp. isolates using five primers, *i.e.*, BAR, BAQ, 18, A9B4 and A9B10. M= DNA marker, Lanes from left to right are *Pestalotiopsis* spp., *P. clavispora* (PS40), *P. microspora* (PS27), *P. neglecta* (PS33), *P. clavispora* (PS42), *P. microspora* (PS31), *P. psidii* (PS3), *P. psidii* (PS9), *P. psidii* (PS19), *P. neglecta* (PS34) and *Pestalotiopsis* spp. (PS23).
*M= 1 kilo base molecular marker.

Table 5. A similarity matrix, in percentage, among the analyzed *Pestalotiopsis* spp. isolates based on RAPD band pattern analysis and Nei & Lis Coefficient.

Isolates	PS40	PS27	PS33	PS42	PS31	PS3	PS9	PS19	PS34	PS23
PS40	100									
PS27	82	100								
PS33	76	85	100							
PS42	78	65	71	100						
PS31	72	89	86	67	100					
PS3	77	82	83	69	85	100				
PS9	78	82	84	62	84	88	100			
PS19	77	79	79	58	75	80	86	100		
PS34	74	86	85	63	87	80	83	80	100	
PS23	60	59	57	55	62	60	60	66	64	100

The RAPD banding patterns were analyzed using UPGMA method to construct a dendrogram (Fig. 8), supported the relationship between the ten analyzed *Pestalotiopsis* spp. isolates and the related *Pestalotiopsis* species. The presence or absence of any particular DNA band was the only factor considered in the computer analysis. The obtained dendogram showed the linkage distance which indicated that the analysed *Pestalotiopsis* isolates were classified into two main clusters. The first cluster A included two sub-clusters, sub-cluster A1 divided into two groups, group1 divided into two sub-groups, group A and group B included isolates PS3, PS9 and PS19 of *P. psidii*. Group 2 divided into three sub-groups, group C and group D included isolates PS33 and PS34 of *P. neglecta*, while group F included isolates PS27 and PS31 of *P. microspora*. Sub-cluster A2 included one group, included isolates PS40 and PS42 of *P. clavispora*. However, the second cluster (B) included an isolate PS23 of *Pestalotiopsis* spp.

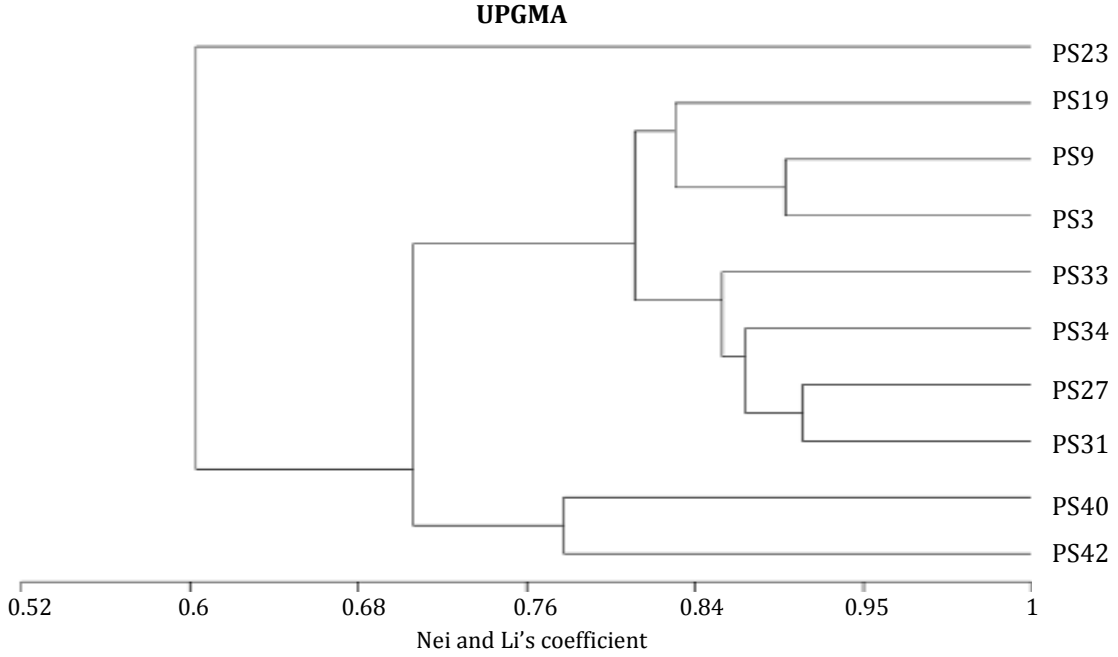

Figure 8. Dendrogram obtained by UPGMA method based on the banding pattern of the RAPD-PCR analysis for ten *Pestalotiopsis* spp. isolates recovered in the survey.

Effects of chitosan on fungal growth and disease reduction

The *in vitro* effect of chitosan on the mycelium growth of *Pestalotiopsis* spp. isolates: Diameter growth of isolates of Pestalotiopsis spp. tested significantly inhibited in vitro with increasing concentration of chitosan determined 5 days after inoculation. Chitosan concentrations of 1.5, 2 and 2.5 % inhibited the mean mycelium diameter growth by 43.88%, 67.56% and 86.53% respectively, compared to 0% inhibition for the

un-amended control, (Table 6). There was a high positive correlation between chitosan concentrations and inhibition of *Pestalotiopsis* isolates with r= 0.98 (Figure 9)**.**

The *in vivo* effect of chitosan for controlling scab of guava: Diameter of scab lesions developed on guava cv. Balady fruits inoculated with Pestalotiopsis spp. isolates significantly decreased with chitosan treatment, at 1.5%, 2% and 2.5% chitosan solutions, compared to the untreated control (Table 7). However, the highest inhibition effect (86.53%) with chitosan was obtained with the 2.5% solution on the fruits (Table 7). Meanwhile a high positive correlation was found between disease reduction and increasing chitosan concentrations with r = 0.94 (Figure 10).

Table 6. Colony diameter and inhibition of *Pestalotiopsis* spp. isolates on PDA amended with different chitosan concentrations.

Treatment	Colony diameter (cm) of different isolates					Mean	Inhibition (%)
	P. psidii (PS9)	*P. neglecta* (PS 33)	*P. microspora* (PS 27)	*P. clavispora* (PS 40)	*Pestalotiopsis* spp. (PS 23)		
Chitosan 1.5%	5.5	5.8	5.6	6.0	5.0	5.00[b]	43.88
Chitosan 2%	3.0	2.9	2.8	2.6	2.2	2.89[c]	67.56
Chitosan 2.5%	1.5	1.1	1.3	1.1	1.1	1.20[d]	86.53
Control 0%	8.8	8.8	8.5	9.0	8.7	8.91[a]	00.00

- Data are means of 4 replicates.

-Values followed by different latter are significantly different at p =0.05

Table 7. Lesion diameter developed on guava fruits (cv. Balady) inoculated with *Pestalotiopsis* spp. isolates and treated with chitosan in different concentrations.

Treatment	Lesion diameter (cm)					Mean	Inhibition (%)
	P. psidii (PS9)	*P. neglecta* (PS 33)	*P. microspora* (PS 27)	*P. clavispora* (PS 40)	*Pestalotiopsis* spp. (PS 23)		
Chitosan 1.5%	1.6	1.9	2.0	2.2	1.6	2.03[b]	36.16
Chitosan 2%	1.5	1.3	1.5	1.1	1.1	1.34[c]	57.86
Chitosan 2.5%	0.7	0.9	0.5	0.5	0.5	0.64[d]	79.87
Control 0%	4.0	3.2	3.5	3.5	2.0	3.18[a]	00.00

** Data are means of 4 replicates.

* Lesion diameters were determined 5 days after treatment.

Values followed by different latter are significantly different at p =0.05

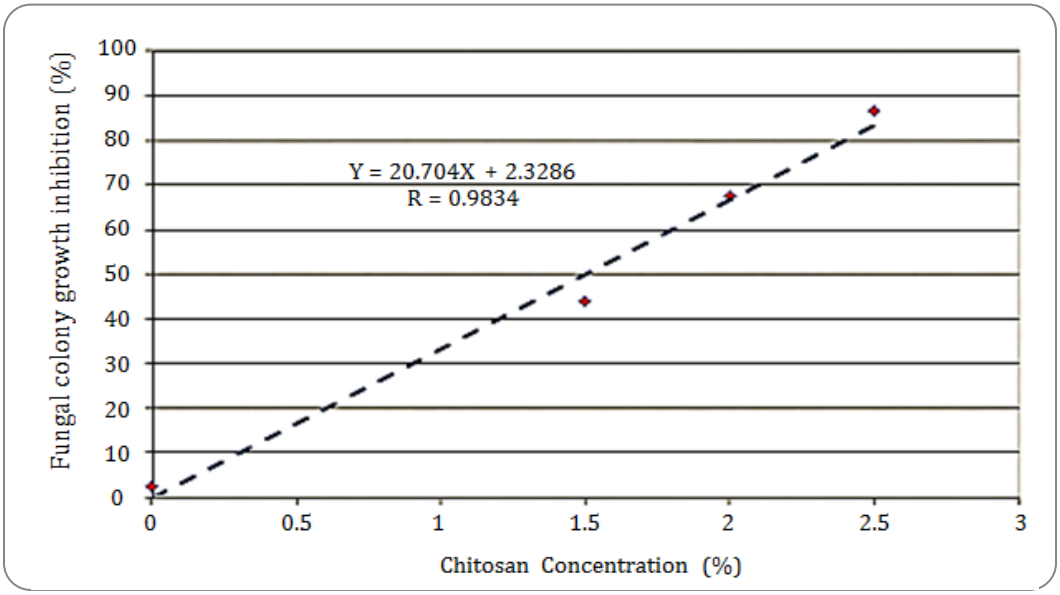

Figure 9. Correlation between different Chitosan concentrations(%) and inhibition of *Pestalotiopsis* spp. colony diameter(cm).

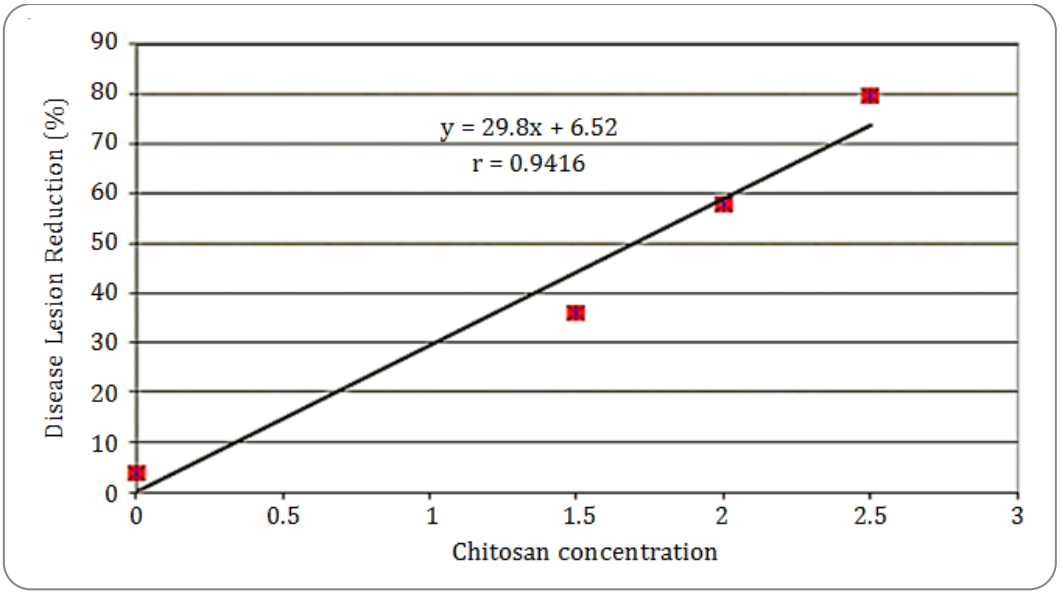

Figure 10. Correlation between disease reduction (%) and different Chitosan concentrations (%).

DISCUSSION

Currently, there is very little information available about the presence, and prevalence, of the scabby canker of guava in Egypt. In the present study, *Pestalotiopsis* spp. were consistently isolated and identified from scabby diseased samples of guava. Forty three isolates of *Pestalotiopsis* spp. were recovered from guava fruits and leaves, showed scab symptoms, collected from different regions in EL-Beheira governorate. The isolates recovered from guava were identified as five species on the basis of the morphological and molecular characterization. The species were *P. psidii*, *P. microspora, P. clavispora, P. neglecta* and *Pestalotiopsis* spp. The *P. psidii* was the most prevalent species and constituted almost half of the total recovered 43 isolates made in the survey. This was followed by *P. microspora* and *P. clavispora* with 9 and 7 isolates, respectively. The *P. neglecta*, however, was confined to only one region (Edkou) out of the four surveyed ones where only four isolates were recovered. These results were in agreement with reports in Hawaii by Keith *et al*., 2006, in Chile by Espinoza *et al*. (2008), in Italy (Ismail & Cirvilleri 2012). More recently, *P. clavispora* causing post-harvest stem end rot of avocado in Chile (Valencia *et al*., 2011), Maharachchikumbura *et al*. (2011). Considered the species as one of the greatest economic importance, since it is pathogenic to several hosts. Also, *P. microspora* causing nut black spot in *Carya cathyensis* (Chuanqing *et al*., 2010). For front view of colony color, P. neglecta and Pestalotiopsis spp, showed white colony color phenotype while it was white to creamy in P. clavispora and P. microspora and creamy in P. psidii. Meanwhile, considerable variations were recorded between isolates of the different species for color of the back view where *P. psidii* colonies were almost yellow to light yellow while *P. clavispora* colonies were of yellowish brown to saffron appearance. *P. neglecta*, however, showed light orange back view for the colonies. Also, *P. psidii*, *P. neglecta* and *P. clavispora* showed big acervulii while *P. microspora* and *Pestalotiopsis spp.* showed small ones. All the isolates recovered were pathogenic to the cv. Balady of guava fruits. However, *P. psidii* isolates were highly virulent while *P. neglecta*, *P. clavispora* showed relatively intermediate virulence. The *P. microspora* and *Pestalotiopsis spp*, however, showed the lowest virulence in this respect. The variation exhibited by the isolates for their virulence could be due to the emergence of region specific virulent strains (Joshi *et al*., 2009). These findings were in harmony with several investigators (Wei & Xu 2004; Liu *et al*., 2007; Jeewon *et al*., 2004, Hu *et al*., 2007 and Maharachchikumbura *et al*., 2011). Meantime, analysis of isolates at the molecular level revealed considerable variations among the identified *Pestalotiopsis* spp. The primers BAR, BAQ, 18, A9B4 and A9B10 were efficient to reveal more variation. Bar marker showed a common band for all *Pestalotiopsis* isolates and species at 500 bp, while BAQ, 18 and A9B4 exhibited banding pattern similar for all isolates of the same species which were different from that of the other

species. The A9B10 marker, however, did not reveal obvious variations among the analyzed isolates. The similarity matrix and the developed dendrogram supported the view that there were five species of *Pestalotiopsis* occurring on guava in the surveyed areas in Egypt as molecular analysis has been always used by several investigators in the identification and classification of *Pestalotiopsis* species (Jeewon *et al*., 2002,2003, 2004; Wei & Xu, 2004; Hu *et al*., 2007; Liu *et al*., 2007; Wei *et al*., 2007; Espinoza *et al*., 2008; Keith, 2008; Luan *et al*., 2008; Karakaya. A,2001; Tejesvi *et al*., 2009; Liu *et al*., 2010; Joshi *et al*., 2009).

Concerning the control of scab on guava fruits, there is an increasing interest for the use of the natural products, such as chitosan, in plant diseases control. The induction of systemic resistance in plants with natural compounds, including chitosan, was a promising approach for plant diseases control (Gozzo, 2003). It has been recognized by the International Commission on Natural Health Products as a natural product for the 21st century (No *et al.,* 2007).In the present study, chitosan significantly inhibited the *in vitro* fungal growth of *Pestalotiopsis* spp. isolates at all concentrations (1.5%, 2%, 2.5%) compared to the control. The *in vivo* studies conducted on guava confirmed the *in vitro* results and the 2.5% chitosan solution was the most effective for scab control. These results were in agreement with Romanazzi *et al*. (2001), No *et al*. (2007), Muñoz *et al*. (2009), Reglinski *et al*. (2010) and Eman El-Argawy (2012). Chitosan is an exogenous elicitor whose activity is due to its polycationic structure and its receptor is a 78 kDa binding protein (Chen & Xu, 2005). However, Faoro *et al*. (2008) and Coqueiro *et al*. (2011), showed that the activity of chitosan was attributed to the accumulation of hydrogen peroxidase in treated tissues, which induces a hypersensitive reaction as a consequence of oxidative microburst and phenolic compound deposition. Meantime, Howe (2005) indicated that chitosan activated jasmonic acid synthesis in treated hosts, while, Aziz *et al*. (2006) indicated that chitosan induced the accumulation of phytoalexins in grape vine leaves, which reduced *Botrytis cinarea* and *Plasmopara viticola* infection. In the present study proved that the antimicrobial characteristics of this substance make it a potential, and moreover, a naturally occurring, food coating and non-toxic for humans (Shaidi *et al*., 1999). Results of the present study confirmed that chitosan offers a safe alternative to synthetic fungicides in postharvest diseases control and could be considered as a potential agrochemical of low environment impact.

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