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

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ABSTRACT

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 acervuli) 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 disease of guava, 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 Pestalotiopsis was early described 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 disease 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
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 Muñoz et al., 2009). It was reported that chitosan confers protection against Botrytis cinerea in Vitis vinifera and controlled grey mould in cucumber 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 governorate, 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 1 min and 1% (v/v) NaOCl (1 min), 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±2°C 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 Monochaeta 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 Pestaotioptis 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 ± 2°C 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 with 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 CGG 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 °C. 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 °C 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).

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-Mahmudy 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 Edkou 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./ Region | *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 \( P. \text{psidii} \) isolates were mostly creamy and none of its recovered isolates was of the white colony phenotype. On the contrary \( P. \text{neglecta} \) colonies were all of the white colony phenotype. However, the two morphology phenotypes were occurred in \( P. \text{microspora} \) and \( P. \text{neglecta} \) in almost equal frequencies (Table 3, Figure 4 & 5).

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

| Characteristics          | Front view | Back view | Acervuli |
|--------------------------|------------|-----------|----------|
|                          | Colony color |          |          |
|                          | Creamy | White | Yellow | L-yellow | Orange | L-orange | Yellowish brown | Saffron | Big | Small |
| \( P. \text{psidii} \)   | 21     | 0     | 11     | 10       | 0      | 0         | 0               | 21      | 0   |
| \( P. \text{microspora} \) | 5      | 4     | 0      | 0        | 5      | 4         | 0               | 0       | 0   | 9    |
| \( P. \text{neglecta} \)  | 0      | 4     | 1      | 0        | 0      | 3         | 0               | 0       | 4   | 0    |
| \( P. \text{clavispora} \) | 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±2°C. 0 = No isolates recorded.
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.

Conidia phenotypes of the recovered *Pestalotiopsis* species: Conidia of *P. psidii* isolates showed 23.09±0.3 μm in length and 4.8±0.4 μm in width. Three apical appendages were 20.48±0.3 μm in length, while the basal
appendages were small hyaline pedicel, of 3.79±0.3 µm 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. clavispora* 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 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±1.4 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. Apical appendages varied from 18.04±1.5 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 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 was 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*. 

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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 & Li's 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   | 64   | 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 | Inhibition (%) |
|---------------|--------------------------------------------|----------------|
|               | *P. psidii* (PS9) | *P. neglecta* (PS 33) | *P. microspora* (PS 27) | *P. clavispora* (PS 40) | *Pestalotiopsis* spp. (PS 23) | Mean   |
| Chitosan 1.5% | 5.5                        | 5.8                        | 5.6                        | 6.0                        | 5.0                        | 5.00b  | 43.88 |
| Chitosan 2%   | 3.0                        | 2.9                        | 2.8                        | 2.6                        | 2.2                        | 2.89c  | 67.56 |
| Chitosan 2.5% | 1.5                        | 1.1                        | 1.3                        | 1.1                        | 1.1                        | 1.20d  | 86.53 |
| Control 0%    | 8.8                        | 8.8                        | 8.5                        | 9.0                        | 8.7                        | 8.91a  | 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) | Inhibition (%) |
|---------------|----------------------|----------------|
|               | *P. psidii* (PS9)    | *P. neglecta* (PS 33) | *P. microspora* (PS 27) | *P. clavispora* (PS 40) | *Pestalotiopsis* spp. (PS 23) | Mean   |
| Chitosan 1.5% | 1.6                        | 1.9                        | 2.0                        | 2.2                        | 1.6                        | 2.03b  | 36.16 |
| Chitosan 2%   | 1.5                        | 1.3                        | 1.5                        | 1.1                        | 1.1                        | 1.34c  | 57.86 |
| Chitosan 2.5% | 0.7                        | 0.9                        | 0.5                        | 0.5                        | 0.5                        | 0.64d  | 79.87 |
| Control 0%    | 4.0                        | 3.2                        | 3.5                        | 3.5                        | 3.0                        | 3.18a  | 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).
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 cathayensis (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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