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Isolation and identification of the strawberry crown rot pathogen

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Abstract

Background: Strawberry is a high-value crop for the economy and nutrition. Five fungal genera were isolated, purified, and identified according to the phenotypic criteria studied: *Pestalotiopsis* spp., *F. solani*, *F. oxysporum*, *Rhizoctonia solani*, and *Macrophomina phaseolina*. **Results**: *Pestalotiopsis* spp. fungi were the most common, accounting for 42.3% of the total isolates. Moreover, *F. oxysporum* was isolated with a frequency of 23.7%, and F. solani with 14.8%. The pathogen isolated from crown root diseased strawberry plants was morphologically similar to the genus Neopestalotiopsis. Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (Pestalotiopsis microspora strain AUMC16335, arrowed) aligned with closely related strains accessed from the GenBank. This strain showed 99.81% identity and 100% coverage with several strains of the same species. A close relationship was also observed between the current strain sequences and some *Pestalotiopsis haikouensis* strains. *Pestalotiopsis microspora* strain (AUMC16336, arrowed) aligned with closely related strains accessed from GenBank. This strain showed 99.81% identity and 100% coverage with several strains of the same species. Five strawberry varieties were evaluated for resistance to Pestalotiopsis. Both Sensation and Fortuna varieties showed a significant increase in the disease incidence rate (DI), which they recorded at 93.33%. The Florida Beauty variety recorded an infection rate of 87.67%. The Winter Star and Festival varieties also recorded the lowest infection rate, with 80.00% and 73.33%.

Keywords: Strawberry, Crown rot, Root rot, *Pestalotiopsis* spp

1. Introduction

Strawberry (*Fragaria x ananassa* Duch.) is a widely favored fruit that can be enjoyed in raw form or through various consumption methods. This perennial herb, belonging to the genus Fragaria within the Rosaceae family, is recognized as the most economically significant cultivated small berry globally [1]**.** Its high nutritional content, favorable economic returns, and short growth cycle contribute to its extensive cultivation across the globe. Strawberries, whether consumed fresh or processed, are the most popular berries due to their appealing appearance, delightful flavor, and health benefits [2 ; 3]**.** Egypt stands as one of the leading producers of strawberries, ranking third after China and the United States. In 2022, the cultivated area for strawberries in Egypt reached 15,836 thousand hectares, with a productivity of approximately 40 thousand tons per hectare, resulting in a total strawberry yield of 638 thousand tons for the year [4]**.**

Fungal diseases significantly impact strawberry cultivation in Egypt, leading to substantial economic losses each year. Various fungi have been identified as responsible for strawberry crown and root rot globally [5]. Among these, Pestalotioid fungi are prominent plant pathogens known to induce leaf spots, fruit rot, and diseases affecting roots and trunks in numerous plant species. *Pestalotiopsis longisetula* (formerly *Pestalotia longisetula*) was first documented as a cause of strawberry fruit rot in Florida in 1972, resulting in considerable losses in certain research plots and commercial fields [6 ; 7]. Subsequently, this pathogen was also found in commercial strawberry plantations in Huelva province, Spain, during the 2013-14 cropping season [8]**.** Additionally, a closely related species, *P. longisetula*, has been recognized as the causative agent of strawberry fruit rot in Egypt [9]**.**
Pestalotiopsis species have been documented about various plant diseases, including the decay of petioles and stolons [10], root rot in the United States [11], and the appearance of necrotic spots on leaves and petioles in Brazil [12]. Additionally, instances of root and crown rot have been reported in Spain [8], and in Belgium, the disease has also been identified in the crown tissues of strawberry plants [13]**.** The characterization of Pestalotiopsis species has primarily focused on morphological characteristics, including conidia size, septation, the presence or absence of appendages, as well as colony texture and color **[**14 ; 15]. Molecular DNA-based techniques have also played a significant role in this characterization [16 ; 17 ; 18]. This was highlighted in the research conducted by Maharachchikumbura *et al.* (2012) [19], which identified ITS, TUB, and translation elongation factor (TEF) as the most effective molecular markers among ten gene regions evaluated for delineating species boundaries within *Pestalotiopsis* spp. Furthermore, a subsequent study by Maharachchikumbura *et al.* (2014) **[7]** led to a multi-locus phylogenetic revision that established two new genera, Neopestalotiopsis and Pseudopestalotiopsis, which, morphologically similar, are phylogenetically distinct from Pestalotiopsis. This research aimed to isolate and identify Pestalotiopsis species that cause strawberry root rot and crown rot diseases.

2. Materials and Methods

2.1. Survey of the disease in different locations in Beheira and Qalyubia Governorates:

A comprehensive survey was carried out to assess the severity of the disease and the infection rate of crown rot in strawberries across various regions of Egypt during the winter growing seasons of 2021-2022. This study focused on Qalyubia Governorate, specifically in several villages such as Al-Deir, Meet Kenana, Arab Al-Ghadiri, Moshtohor, and El-Hsania. Additionally, it included the Beheira Governorate, examining two centers within the governorate: Badr and Kom Hamada. The objective was to investigate the prevalence of crown rot in strawberry farms situated in diverse locations with varying soil types across both governorates. A total of one hundred and eighty samples were collected from different sites during the strawberry growing season in each governorate.

2.2. Isolation, purification, and identification of the pathogen:

Plants showing symptoms of root rot and crown rot disease were collected from different places in Qalyubia Governorate (Al-Deir, Meet Kenana, Arab Al-Ghadiri, and El-Hsania) and in the Beheira Governorate from the Badr Center, which includes the areas of Salah El-Din, Om Sabr, Omar Shahen, Omar Makram, Badr. and the center of Kom Hamada, which includes the areas of EL Zafrn, El Tayarya, Manshit Abo Raya, Kafr Zyada, and Waqed.

2.2.1- Isolation of the pathogen:

Strawberry plants exhibiting symptoms were gathered from the Qalyubia and Beheira governorates. Observations of symptoms were made, and samples were collected from both symptomatic and seemingly healthy plants. These samples were meticulously prepared and stored in an icebox until they reached the laboratory. The roots were thoroughly washed under running water to eliminate any adhering debris and contaminants before being surface sterilized with a 1% sodium hypochlorite solution for 1 to 2 minutes. Following this, the samples were rinsed with sterilized distilled water for 2 minutes and then placed between sterile filter paper to facilitate drying. Using a sterile medical scalpel, the samples were then cut. The affected trunk and crown regions were sectioned into pieces measuring 3 to 5 mm. These segments were placed in 9 cm Petri dishes containing potato dextrose agar (PDA) supplemented with the antibiotic streptomycin at a concentration of 0.1 g/L and subsequently incubated at a temperature of 25±2°C for 10 days. Fungal hyphae emerging from the edges of the tissue were aseptically transferred to new PDA plates and incubated at 25 ± 2°C for 7 days. Monocultures were established following the method described by Zhang (2012) [20]. Following a brief duration, the fruiting bodies are subjected to crushing in order to isolate the spores and create a spore suspension. This suspension is then inoculated onto fresh PDA plates, where the germinated conidia are aseptically transferred to additional PDA plates. The purified cultures are preserved in tubes containing PDA, which are sealed with sterile mineral oil and maintained at 20°C, while also being stored at 4°C to ensure their viability for future research.

2.2.2. Purification of isolated fungi using the single conidial technique:

A conidial suspension from fungal isolates was created by adding 50 mL of sterilized distilled water to the surface of the agar. A sterile needle was employed to scrape the conidia, which were then thoroughly mixed with water to produce a series of dilutions. Subsequently, the spore suspension was introduced into Petri dishes containing sterile solid PDA [21]. The plates were incubated until the emergence of germ tubes. The spores were then transferred to PDA medium in slanted test tubes and Petri dishes using a flat-ended needle, with the inoculated plates being incubated at 25°C for a duration of 7 days.

2.2.3. Purification of isolated fungi using hyphal tip technique:

Pure cultures were achieved by isolating the hyphal tips of non-sporulating fungi, which were carefully marked and excised with a sterilized, flamed, flat-ended needle. The excised tips were then placed onto PDA medium and incubated at 25°C for a duration of 7 days for subsequent analysis [21]. The initial identification of these fungal colonies was conducted based on specific morphological characteristics [7 ; 19].

2.2.4-Pathological tests:

Four isolates of *Pestalotiopsis* fungus were tested for their ability to cause diseases under greenhouse conditions at PICO Modern Agriculture, Beheira, on Sensation variety from PICO Modern Agriculture strawberry nursery, Beheira, Egypt during the 2022-2023 season.

2.3.1. Soil sterilization:

The sandy clay soil [2 clay: 2 sand w/w] was sterilized by mixing it well with a commercial 5% formalin solution (one liter of 5% formalin solution/cubic foot of soil mixture) and covered with polyethylene for 2 weeks, and then the cover was removed. Stir and aerate the soil several times for 15 days until the formalin evaporates. Likewise, plastic seedling bags (23 cm) were sterilized by soaking them in a commercial 5.0% formalin solution for 20 minutes and leaving them to dry for 24 hours. Then fill it with previously sterilized soil [22].

2.3.2. Preparation of inoculum and soil infestation:

Following the isolation and identification of the fungus *Pestalotiopsis microspora* strain AUMC 16335, the initial cultivation was conducted on a solid PDA medium. After the medium was prepared, sterilized, and poured into sterile dishes, these dishes were incubated at room temperature for a duration of 10 days. The inoculum was subsequently prepared using rice as the growth substrate; this involved taking one kilogram of rice, placing it in a thermal bag, and adding 400 mL of sterile distilled water to soak for 30 minutes. The thermal bag was then secured with a cotton plug and subjected to autoclaving at a temperature of 121°C for 15 minutes. Following this process, the bags were inoculated with 10 discs

of 10-day-old fungal inoculum. The bags were then allowed to incubate at room temperature for 21 days before being placed in an electric dryer set to 30°C [23].

2.3.3. Pathogenicity test:

Healthy Sensation strawberry seedlings were cultivated following a meticulous process. Initially, the seedlings underwent sterilization by being submerged in a 2.0% sodium hypochlorite solution for a duration of two minutes. Subsequently, they were rinsed multiple times with sterile tap water before being planted directly into plastic bags with a diameter of 23 cm, which were filled with a soil mixture that had been sterilized using formalin. The fungus *Pestalotiopsis microspora* was introduced at a concentration of 2 grams of powder (with each gram containing 1 x 10^6 spores) per kilogram of the soil within the plastic seedling bags. As a negative control, only plastic seedling bags containing sterilized soil were utilized. The bags were maintained for one week, ensuring a humidity level of 50-60% through regular watering. Each variety was subjected to inoculation with the fungal isolates, with 15 replicates established for each, including 15 replicates designated as negative controls. All plants were kept in a greenhouse environment, maintained at a temperature of 25±2°C and a relative humidity of 50–60%. Sixty days post-planting, the extent of disease severity on the plants was assessed.

2.3.4. Disease assessment:

The planted strawberry cultivar that was tested eight weeks after planting was used to gauge the severity of the fungal isolates. After removing the remaining seedlings, they were cleaned with running water and sterile water before being diagnosed. Using a severity scale from 0 to 4, as proposed by McKinney (1923), to assessment the disease on the leaf portion of strawberry plants:

Where: 0= healthy plant, 1 = beginning of wilt symptoms, 2= pronounced wilt symptoms, 3 = majority of leaves wilted/dead, plants generally very small, 4 = dead plant. The decline/death scores recorded along each bed at each field were converted into the decline/death indices.

The discoloration of the vascular tissue harvested from the crown was also examined, a longitudinal section was made, and the severity of the disease, the crown, and roots were evaluated separately based on the disease severity scale from 0 to 4 following [24]. where: -

0 = no crown/root tissue discolored.

1=<25% crown/root tissue discolored.

2=≥25, <50% crown/root tissue discolored.

3 = ≥50, <75% crown/root tissue discolored.

4=≥75% crown/root tissue discolored.

Also, Disease severity and disease incidence was recorded for each treatment by the equations:

$$\%\,DS = \frac{[(a\,x\,0)+(b\,x\,1)+(C\,x\,2)+(d\,x3)+(e\,x4)]}{[\,(a+b+c+d+e)\,x\,4)\,]}\,x\,100$$

$$\%\ \text{Disease incidence} = \frac{\text{plants infected of number}}{\text{plants of number total}}\ x\ 100$$

2.4-Molecular identification of fungal isolates

Fungal isolates were cultivated on potato sucrose agar (PSA) in 9mm plates and incubated at a temperature of 28° C for 7 days, as described by [25]. A small portion of the fungal growth was collected, suspended in 100 µL of sterile distilled water, and subjected to boiling at 100° C for 15 minutes. These samples were subsequently forwarded to SolGent Company in Daejeon, South Korea, for the processes of DNA extraction, PCR, and rDNA sequencing. The extraction and isolation of fungal DNA were conducted using SolGent purification beads. The internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA (rRNA gene) were amplified with the universal primers ITS-1 forward (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS-4 reverse (5' - TCC TCC GCT TAT TGA TAT GC - 3'). Amplification was done via polymerase chain reaction (PCR) using an ABI 9700 thermal cycler. The resulting PCR products were purified utilizing the SolGent PCR Purification KitUltra before proceeding to sequencing. The purified products were verified through electrophoresis on a 1% agarose gel with a size marker. The bands were then eluted and sequenced. Each sample underwent sequencing in both forward and reverse directions using the same primer pair, incorporating dideoxynucleotides (ddNTPs) as per the recommendations of [26]. Contigs were generated from the sequence data using the CLCBio Main Workbench software. The sequences obtained from each isolate were further analyzed using the BLAST tool available on the National Center of Biotechnology Information (NCBI) website. The sequences acquired, along with those sourced from the GenBank database, were subjected to Clustal W analysis using MegAlign software version 5.05 for phylogenetic assessment.

2.5. Varietal reaction:

Five strawberry varieties (Festival, Fortuna, Sensation, Beauty, and Winter Star) were evaluated in terms of their susceptibility to crown rot disease caused by the fungus *Pestalotiopsis* (P-2) under greenhouse conditions at a temperature of 25 ± 2 °C. The tested varieties were obtained from commercial agricultural nurseries of the Pico Modern Agriculture Company, Beheira, Egypt. Most virulent strains of the fungus *Pestalotiopsis* (P-2) was used. A rate of 2 grams of pathogen inoculum per kilogram of soil (w/w) was used individually in soil infestation. The inoculum is mixed well with the soil and watered regularly for a week before planting. Each seedling was planted in seedling bags with a diameter of 23 cm. Each cultivar was planted similarly under greenhouse conditions, and 15 replicates were made for each cultivar. Bags containing only sterile soil were used as a control, and 15 replicates were made for each specific treatment. Plants were watered and agricultural practices were performed as needed. The experiment was designed in a completely randomized design. 60 days after planting, disease incidence and disease severity were calculated as previously described.

3. Results

1. **Frequency of the isolated fungi recovered from Strawberry plants infected with**
2. **crown rot disease in different locations in the Qalyubia and Beheira governorates:**

Strawberry plants are considered infected with crown rot when the initial symptoms of crown rot appear, which usually occurs after the plants begin to flower, as the above-ground symptoms initially range from drying of the leaf edges. (Figure1- A) Until the leaves and flowers dry completely as the disease progresses, wilting and stunting of the plant also occur, leading to the collapse and death of the entire plant in the end, while underground symptoms are characterized by black discoloration of the roots and the appearance of brown-orange in the internal tissues of the crown (Figure1- B), which contributes to plant stunting, wilting and collapse of infected plants compared to healthy plants.

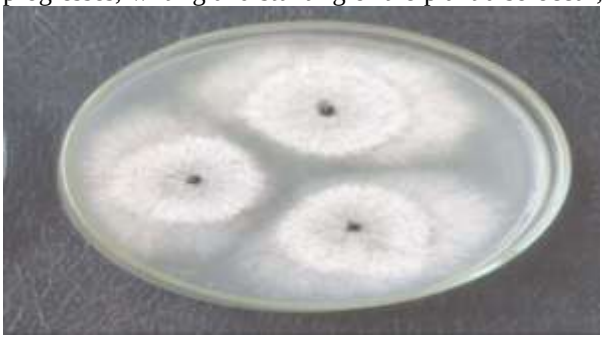

Fig. 1. Symptoms of strawberry crown rot disease

Fig. 2. *Pestalotiopsis* spores and the growth on Petri dishes

1.2. Observing symptoms and the isolated fungi:

Strawberry plants with the previous symptoms on the five strawberry varieties were collected from the five locations (Meet Kenana, Al-Deir, Arab Al-Ghadiri, Moshtohor, and El-Hsania) in Qalyubia Governorate, Egypt. Five fungal genera were isolated, purified, and identified according to the phenotypic criteria studied: *Pestalotiopsis* spp., *F. solani, F. oxysporum, Rhizoctonia solani,* and *Macrophomina phaseolina. Pestalotiopsis* spp. fungus was the most common, accounting for 42.3% of the total isolates. *F. oxysporum*

was isolated with a frequency of 23.7% and F. *solani* with a frequency of 14.8%. Whereas, for *R. solani* and *Macrophomina phaseolina* were 10.3 and 9.8%, respectively as in Table 1. Deformed crown tissues were also collected from the five locations (Salah al-Din, Umm Saber, Omar Shaheen, Omar Makram, and Badr) in the Badr- Center, Al-Buhaira. The most common fungus isolated was *Pestalotiopsis* spp, with a percentage of 48.86%. *F. oxysporum* was isolated repeatedly, with a percentage of 20.6%. *F. solani* with a frequency of 12.5%, however, *R. solani* and *Macrophomina phaseolina* are 9.09% and 9.09%, respectively (Table 2). Also, the most common fungus in the five locations (Saffron, Al-Tairiya, Mansheyet Abu Rayya, Kafr Ziadeh, and Waqed) in the Kom Hamada-Beheira center was *NeoPestalotiopsis* with a rate of 44.14%, followed by *F. oxysporum* with a rate of 24.32%. *F. solani* with a frequency of 14.51%, whereas *Rhizoctonia solani* and *Macrophomina phaseolina* were 10.81% and 7.22%, respectively, as in **Table 3**.

Table 1. Frequencies of the isolated fungi recovered from strawberry plants infected by root and crown rot in Qalyubia governorate, Egypt.

Location	Meet Kenana		Al-Deir		Arab Al- Ghadiri		Moshtohor		El-Hsania		Total	
Isolate	**No.**	**Frequency %**	**No.**	**Frequency %**	**No.**	**Frequency %**	**No.**	**Frequency %**	**No.**	**Frequency %**	**No.**	**Frequency %**
Pestalotiopsis spp.	16	45.7	13	41.92	11	39.28	9	37.5	8	47	57	42.3
F. oxysporum	9	25.7	7	22.58	5	17.86	6	25	5	29.4	32	23.7
F. solani	4	11.4	4	12.9	6	21.43	4	16.7	2	11.8	20	14.8
R. solani	3	8.6	4	12.9	4	14.29	2	8.3	1	5.9	14	10.3
Macrophomina phaseolina	3	8.6	3	9.7	2	7.14	3	12.5	1	5.9	12	8.9
Total	**35**	**100**	**31**	**100**	**28**	**100**	**24**	**100**	**17**	**100**	**135**	**100**

Table 2. Frequencies of the isolated fungi recovered from strawberry plants infected by root and crown rot in Badr District, El-Behira Governorate.

Location	Salah El din		Om Sabr		Omar Shahen		Omar Makram		Badr		Total	
Isolate	No.	Frequency %	No.	Frequency %	No.	Frequency %	No.	Frequency %	No.	Frequency %	No.	Frequency %
Pestalotiopsis spp.	7	50	8	53.33	11	47.83	9	52.94	8	42.12	43	48.86
F. oxysporum	3	21.43	3	20	5	21.74	4	23.53	3	15.78	18	20.46
F.solani	1	7.14	2	13.33	3	13.04	2	11.77	3	15.78	11	12.5
R. solani	1	7.14	1	6.67	2	8.70	1	5.88	3	15.78	8	9.09
Macrophomina phaseolina	2	14.29	1	6.67	2	8.70	1	5.88	2	10.54	8	9.09
Total	14	100	15	100	23	100	17	100	19	100	88	100

Table 3. Frequencies of the isolated fungi recovered from strawberry plants infected by root and crown rot in Kom Hamada District, El-Behira Governorate.

Location	EL Zafrn		El Tayarya		Manshit Abo Raya		Kafr Zyada		Waqed		Total	
Isolate	No.	Frequency %	No.	Frequency %	No.	Frequency %	No.	Frequency %	No.	Frequency %	No.	Frequency %
Pestalotiopsis spp.	11	47.83	9	45	10	41.7	8	44.4	11	42.31	49	44.14
F. oxysporum	6	26.08	5	25	4	16.7	5	27.8	7	26.92	27	24.32
F. solani	3	13.04	2	10	5	20.8	2	11.11	3	11.54	15	13.51
R. solani	1	4.35	3	15	3	12.5	1	5.58	4	15.38	12	10.81
Macrophomina phaseolina	2	8.7	1	5	2	8.3	2	11.11	1	3.85	8	7.22
Total	23	100	20	100	24	100	18	100	26	100	111	100

1.3. Isolation of fungi causing crown rot disease from strawberry plants:

1.1. Isolation of the fungus *Pestalotiopsis* causing crown rot:

As shown in **Table 4,** the organism that causes crown rot disease, Pestalotiopsis, was isolated from strawberry plant samples that showed symptoms of crown rot, which were collected from different locations in the Beheira Governorate, Egypt, including the Kom Hamada and the Badr Centers, and the Qalyubia Governorate, Egypt, including the Tukh and Shibin El Qanater Centers.

Table 4: Isolation of the fungus causing *Pestalotiopsis* crown rot from the Egyptian governorates of Beheira and Qalyubia.

Governorate	Location	Isolate No.
Beheira	Badr	P-1
	Kom Hamada	P-2
Qalyubia	Tukh	P-3
	Shibin El Qanater	P-4
Control		-----

3. Pathogenicity test:

Table 5: Pathogenicity test of the *Pestalotiopsis* isolates:

3.1. Testing the pathogenicity of the four *Pestalotiopsis* isolates:

A pathogenicity test was conducted for four *Pestalotiopsis* isolates under greenhouse conditions on the Sensation cultivar.

Data in **Table 5** indicate that all *Pestalotiopsis* isolates were pathogenic to the tested plants and caused symptoms of crown rot disease. The highest pathogenic isolate was (P-2), which was isolated from the Beheira (Kom Hamada Center), where it caused the highest rate of disease incidence (DI%) of 100% and disease severity (DS%) on the Foliar part and vascular tissues of 86.7% and 83. 3%, respectively, followed by an isolate (Badr Center). Where the disease incidence (DI%) was 93.3%, and the disease severity (DS%) on foliar part and vascular tissues was 80.0 and 76.67%, respectively. The Qalyubia isolate (Shibin El Qanater Center) recorded a disease incidence rate (DI%) of 73.3% and disease severity (DS%) on foliar part and vascular tissue reached 61.7% for each of them. On the other hand, the lowest DI% was recorded in the Qalyubia isolate (Tukh Center) reaching 66.7%, and DS% on foliar part and vascular tissues (56.7 and 53.3%), respectively.

Governorate	Location	Isolate No.	DI%	DS%	
				Foliar part	Vascular tissue
Beheira	Badr	P-1	93.3	80.0	76.67
	Kom Hamada	P-2	100	86.7	83.3
Qalyubia	Tukh	P-3	66.7	56.7	53.3
	Shibin El Qanater	P-4	73.3	61.7	61.7
Control		-----	0.0	0.0	0.0

4. Sequencing results:

Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (*Pestalotiopsis microspora* strain AUMC16335, arrowed) aligned with closely related strains accessed from the GenBank. This strain showed 99.81% identity and 100% coverage with several strains of the same species. A close relationship was also observed between sequences of the current strain and some strains of *Pestalotiopsis haikouensis.*

As for *Pestalotiopsis microspora* strain AUMC16336, arrowed) aligned with closely related strains accessed from GenBank. This strain showed 99.81% identity and 100% coverage with several strains of the same species. A close relationship was also observed between the current strain sequences and some *Pestalotiopsis haikouensis* and *Neopestalotiopsis formicarum* strains.

ITS sequences of sample TA-1: *Pestalotiopsis microspora* strain AUMC 16335 (518 letters)

GGAGGGATCATTATAGAGTTTTCTAAACTCCCA
ACCCATGTGAACTTACCTTTTGTTGCCTCGGCA
GAAGTTATAGGTCTTCTTATAGCTGCTGCCGGT
GGACCATTAAACTCTTGTTATTTTATGTAATCT
GAGCGTCTTATTTTAATAAGTCAAAACTTTCAA
CAACGGATCTCTTGGTTCTGGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCATTAGTATTCTAGTGGGCATGC
CTGTTCGAGCGTCATTTCAACCCTTAAGCCTAG
CTTAGTGTTGGGAATCTACTTCTCTTAGGAATT
GTAGTTCCTGAAATACAACGGCGGATTTGTAGT
ATCCTCTGAGCGTAGTAATTTTTTTCTCGCTTTT
GTTAGGTGCTATAACTCCCAGCCGCTAAACCCC
CAATTTTTTGTGGTTGACCTCGGATCAGGTAGG
AATACCCGCTGAACTTAAGCATA

ITS sequences of sample TA-2: *Pestalotiopsis microspora*, strain AUMC16336 (524 letters)

CTGCGGAGGGATCATTATATAGTTTTCTAAACT
CCCAACCCATGTGAACTTACCTTTTGTTGCCTC
GGCAGAAGTTATAGGTCTTCTTATAGCTGCTGC
CGGTGGACCATTAAACTCTTGTTATTTTATGTA
ATCTGAGCGTCTTATTTTAATAAGTCAAAACTT
TCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCCATTAGTATTTTAGTGGGC
ATGCCTGTTCGAGCGTCATTTCAACCCTTAAGC
CTAGCTTAGTGTTGGGAATCTACTCCTTTTATT
AGTTGTAGTTCCTGAAATACAACGGCGGATTTG
TAGTATCCTCTGAGCGTAGTAATTTTTTTCTCGC
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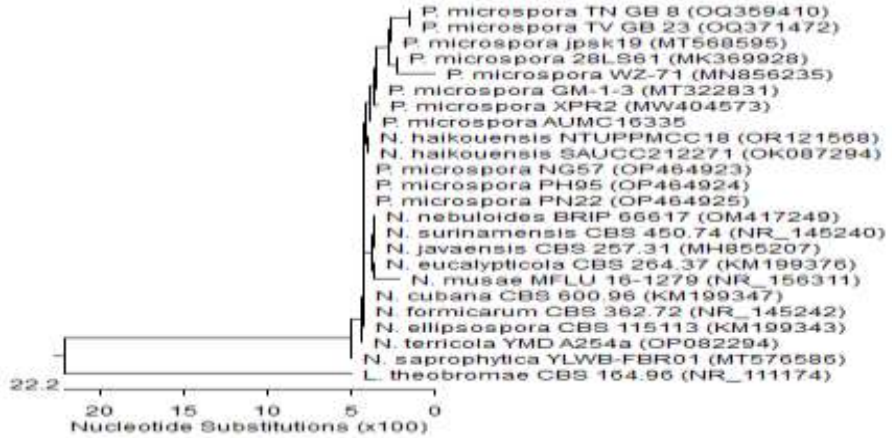
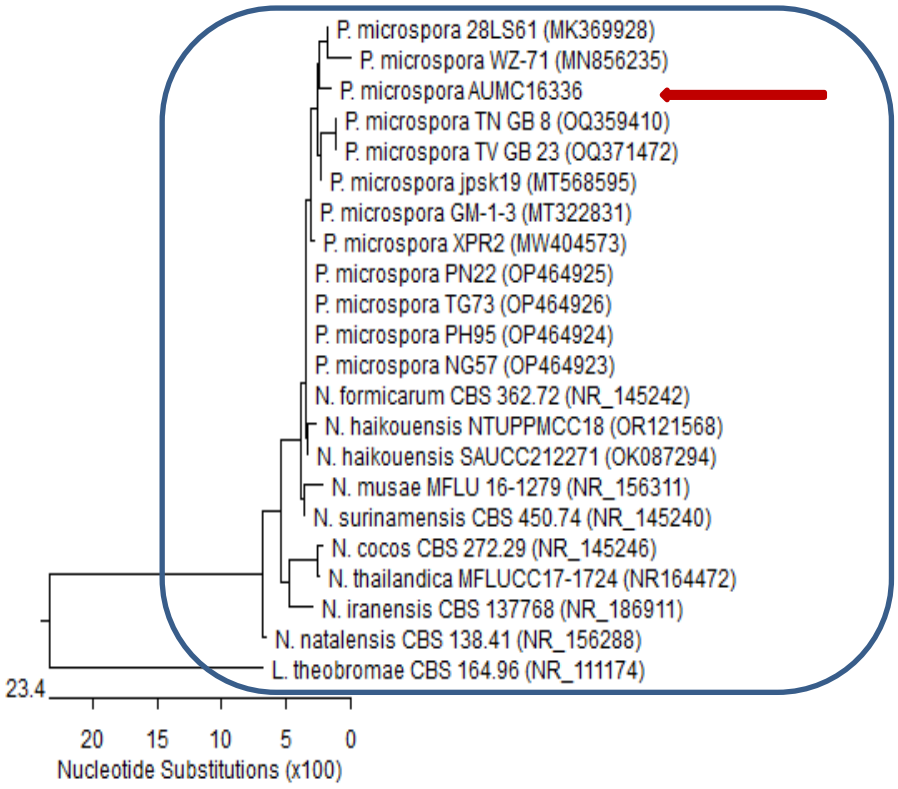


Fig. 3: Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (*Pestalotiopsis microspora* strain AUMC16335, arrowed) aligned with closely related strains accessed from the GenBank. The outgroup strain was represented by *Lasiodiplodia theobromae. L.*.=*Lasiodiplodia*, N.= *Neopestalotiopsis* and *P*.= *Pestalotiopsis*



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Optional tree including the two strains isolated in the present study

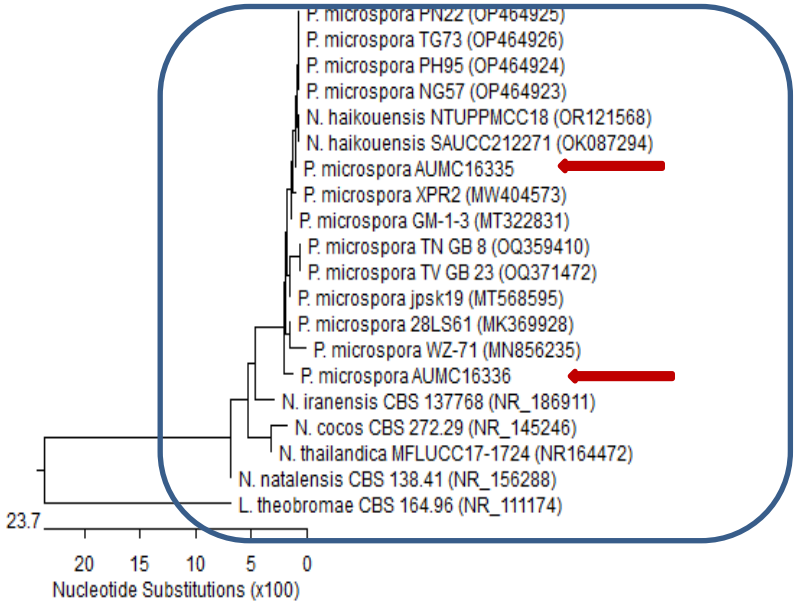

Fig.5: Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (*Pestalotiopsis microspora* strain AUMC16335 and *P. microspora* strain AUMC16336, arrowed) aligned with closely related strains accessed from the GenBank. The outgroup strain was represented by *Lasiodiplodia theobromae. L.*=*Lasiodiplodia*, N.= *Neopestalotiopsis* and *P*.= *Pestalotiopsis*.

3.1. Interactions of some strawberry varieties with crown rot disease:

Five strawberry varieties were tested for resistance or sensitivity to the most virulent strains of the fungus *Pestalotiopsis* (P-2) under greenhouse conditions. The

data in Table 6 show that all tested strawberry varieties were infected with crown rot disease, as they showed symptoms similar to symptoms observed in the field, which is the beginning of infection with the fungus *Pestalotiopsis*. As the infection progresses, leaves dry completely and end with the death of the entire plant, as shown in Figure 1-A). There is also a change in the color of the internal tissues of the crown Figure 6. *P. microspora* was then successfully re-isolated from symptomatic tissue. Both Sensation and Fortuna varieties showed a significant increase in the disease incidence rate (DI), as they both recorded 93.33%. The Florida Beauty variety recorded an infection rate of 87.67%. The Winter Star and Festival varieties also recorded the lowest infection rate, as they recorded 80.00% and 73.33%, respectively. The Disease severity (DS) was recorded two months after inoculation with the fungus for different strawberry varieties. The highest rate of infection severity was recorded on the shoots of the Sensation variety with 56.67%, followed by the Florida Beauty variety with a rate of 48.33%. The Fortuna and Winter Star varieties also recorded 46.67% and 41.67%, respectively. The lowest rate of infection severity was recorded on the Festival variety, with a rate of 36.67%. The highest rate of infection severity on the internal tissues of the crown was for the Sensation variety, at a rate of 58.33%, followed by the Florida Beauty Pence variety, with 53.33%. Both the Fortuna and Winter Star varieties recorded a rate of (DS) of 46.67% and 41.67%, respectively. However, the lowest registration of DS was 40% for festival variety.

Fig. 6: Interactions of some strawberry varieties with crown rot disease

Table 6. Varietal reactions of some strawberry varieties to crown rot infection under greenhouse conditions.

Cultivar	Disease incidence %	Disease severity (DS) %	
		Foliar part	Vascular tissue
Festival	73.33	36.67	40.00

Fortuna	93.33	46.67	46.67
Sensation	93.33	56.67	58.33
Winter star	80.00	41.67	41.67
Florida Beauty	86.67	48.33	53.33
Control	6.67	6.67	0.00

Discussion

Five genera of fungi were isolated, purified, and identified based on the studied phenotypic criteria: *Pestalotiopsis* spp., *F. solani*, *F. oxysporum*, *Rhizoctonia solani*, and *Macrophomina phaseolina*. Among these, Pestalotiopsis spp. was the most prevalent, representing 42.3% of the total isolates. Numerous studies have suggested that *Pestalotiopsis* species are typically not host-specific and can be found in association with a diverse array of hosts and substrates [16]. **(**The earliest study of [27] **McQuilken and Hopkins (2004)** stated that *P. sydowiana (Bresad) serves as a causal agent for the stem-base and root diseases affecting ericaceous crops, such as Calluna, Erica, Pieris, and Rhododendron, in nurseries across the United Kingdom.* A recent study of **Grantina-Ievina and Kalnina (2016)** [28]. reported that *Pestalotiopsis* spp. was frequently isolated from 5% to 42% of the strawberry plants assessed, all of which displayed symptoms of crown rot disease. [29]. research has empirically determined that *Pestalotiopsis* spp. may be responsible for causing crown rot disease in strawberry plants in Belgium. Moreover, **Ara *et al.* (2017)** [30]. reported that *Pestalotiopsis* spp. is the causative agent of crown rot disease in strawberries in Bangladesh. Consequently, our findings are in agreement with those earlier observations. The isolation frequency of *F. oxysporum* was noted to be 23.7%, whereas *F. solani* was isolated with a frequency of 14.8%. These results agree with the results obtained by **Essa *et al,* (2018**) [34]. They *found that F. oxysporum* and *R. solani* were associated with crown and root rot, but isolated at low frequencies of 17.38 and 9.59 %, respectively. Similarly, **Grantina-Ievina Kalnina (2016)** [28]. stated that *Fusarium oxysporum* and *Rhizoctonia solani* were identified as pathogens associated with crown rot in 5-30% and 8-22% of the studied plants, respectively. The classification of the Pestalotiopsis genus is complicated, as it faces significant challenges in species-level identification due to the extensive morphological variation present [32]. **(Karakaya 2001).** *Neopestalotiopsis rose* exhibited minimal variation in both colony and conidia morphology, appearing quite similar to one another. Molecular marker tools were instrumental in distinguishing and clarifying species boundaries within the genus Pestalotiopsis. The phylogenetic tree constructed from the ITS sequences of rDNA for the fungal sample isolated in this study (*Pestalotiopsis microspora* strain AUMC16335, indicated by an arrow) aligned closely with related strains retrieved from GenBank. This particular strain demonstrated a 99.81% identity and 100% coverage with multiple strains of the same species. Additionally, a close relationship was noted between the sequences of the current strain and certain strains of *Pestalotiopsis haikouensis*. Similarly, *Pestalotiopsis microspora* strain AUMC16336 (also indicated by an arrow) aligned with closely related strains from GenBank, showing a 99.81% identity and 100% coverage with several strains of the same species. A close relationship was also identified between the sequences of this strain and some strains of Pestalotiopsis haikouensis and Neopestalotiopsis formicarum. These results are in harmony with those obtained by **Essa *et al,* (2018)** [34]. The identification of Pestalotiopsis fungal isolates was accomplished through the amplification and sequencing of the ITS, TEF-1α, and β-tubulin gene regions. A BLASTn search conducted in the GenBank database indicated that all sequences obtained demonstrated a similarity ranging from 99% to 100% with *Neopestalotiopsis rosae*. Moreover, **Mahapatra *et al.,* (2018)** [35]. reported that the pathogen isolated from strawberry plants exhibiting crown root disease exhibited morphological characteristics closely resembling those of the genus Neopestalotiopsis, which has been previously documented in other countries. Molecular characterization confirmed that the pathogen identified in this study is *Neopestalotiopsis clavispora* (NCBI accession number MF377347). An assessment of five strawberry varieties for their resistance to Pestalotiopsis revealed that the Sensation and Fortuna varieties experienced a notable rise in disease incidence, with the Florida Beauty variety following closely behind. Moreover, the Winter Star and Festival varieties recorded the lowest infection rate. These results agree with [36]. **(Sanchez *et al.,* 2016).** They reported that among the eleven strawberry varieties, Festival', 'Amiga', and 'Naiad' were the least susceptible varieties.

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