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ORIGINAL ARTICLES

Species identification of Colletotrichum the causal agent of strawberry anthracnose and their effects on fruit quality and yield losses

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ABSTRACT

This study was conducted during the period from 2010 to 2012 and focused on strawberry anthracnose associated with diseased strawberry plants which caused root rot fruit and yield losses by *Colletotrichum* spp. Two local species of *Colletotrichum* fungus were identified based on the morphological, pathological characteristics and PCR analyses using specific primers. The first species was identified as *Colletotrichum gloeosporioides* [teleomorph *Glomerella cingulata*], and the second was identified as *C. acutatum* [teleomorph *Glomerella acutata*]. Pathogenicity was tested and confirmed by artificial inoculation of four strawberry cvs. i.e. Festival, Sweet Charlie, Tamar and Yael. Both two local species were found to be pathogenic for both tested strawberry cultivars and all inoculated parts showed typical symptoms of anthracnose. Tamar cv, was the most susceptible than Yael cv. Also, data indicated that, *C. gloeosporioides* was more virulence than *C. acutatum* in pots grown under greenhouse conditions. Fruits of three cultivars i.e. Sweet Charlie, Tamar and Yael were tested for their susceptibility to *Colletotrichum acutatum* and *C. gloeosporioides.* Results indicated that, both species were found to be pathogenic to all tested strawberry fruit cultivars. Yael cv, was the most susceptible than Tamar fruits. and *C. gloeosporioides* was more virulent than *C. acutatum.* The former data led to dramatic changes of all morphological, physical and bio-chemical properties in addition to some mineral contents of inoculated fruits compared with non-inoculated ones. This is the first report, relying on accurate molecular identification (PCR), of the two major *Colletotrichum* species isolated from strawberry plants in Egypt.

Key words: Strawberry, Anthracnose**,** Polymerase Chain Reaction (PCR), Fruit rot, Growth, Yield, Quality characteristics.

Introduction

Strawberry (*Fragaria x ananassa* Duch), is one of the most important vegetable crops for local consumption and exportation. Strawberries are an excellent source of vitamin C, since they contain an average 40–90 mg of vitamin C. This means that with a supply of 100g of strawberries, the daily needs of vitamin C will be covered (Berta, *et al.*, 2008). The major sugars in strawberry fruit are sucrose, glucose, and fructose, accounting for more than 99% of the total sugars in ripe fruit (Agulheiro and Barreto, 2008). The soluble solids and total sugars contents of strawberries tend to increase as the fruit matures (Perez, *et al*., 2008).

Fungi are the major microorganisms attacking strawberry plants and causing severe diseases at different developmental stages. Anthracnose refereed to several species of the fungus *Colletotrichum.* This is one of the most important diseases of strawberry and well known in most of the producing countries. Thus anthracnose can cause heavy losses in strawberry grown for processing which are left to fully ripe stage on the plant. Plants are susceptible to infection at all stages of growth, young tissue being especially vulnerable (Snowdon, 1990).

Colletotrichum acutatum, C. fragaria and *C. gloeosporioides (Glomerella cingulata)* are the major Colletotrichum species to cause crown rot in strawberry plant (Denoyes - Rothan *et al.*, 2003). *Colletotrichum acutatum* is a quarantine organism on strawberries in the EU, in importing strawberry plants. The disease can occur on all parts of the plant, including the crown and roots, but it is primarily a disease of ripe fruits. *Colletotrichum gloeosporioides* [*Glomerella cingulata*] causes a serious crown rot of strawberry and some isolates from native plants are pathogenic to strawberry. In a previous study, typical anthracnose symptoms were observed in cultivated strawberry fields in Kalubia and Ismailia governorates, Egypt. (Embaby *et al.*, 2010).

Also, Fruit rot disease caused by fungi is one of the major problems to strawberry cultivation and production reducing their quantity and quality and causing economic losses in the field, at harvest time, during marketing and exportation. *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea, Rhizopus stolonifer, Rhizoctonia solani*, *Phytophthora cactorum*, *Fusarium* spp., *Penicillium* spp. and *Sclerotinia sclerotiorum* are the most

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fungal isolates causing strawberry fruit rots in Egypt (Khafagi, 1982; Tadrous, 1991 and Tarek, 2004). In addition to *Pestalotia longisetula* (Embaby, 2007 a & b).

Moreover, other fungi were isolated from different locations all over the world. *Aspergillus, Botrytis, Colletotrichum, Geotrichum, Mucro, Penicillium, Pestaliopsis, Phytophthora* and *Rhizopus stolonifer* were found to infect the crop (Fraire-Cordero, *et al.*, 2003 and Paivi, *et al.*, 2008).

Colletotrichum spp. cause a wide range of strawberry diseases ranging from crown-root rot, and fruit infection resulted in yield losses (Freeman and Katan 1997 and Garrido, *et al.*, 2008). *Colletotrichum acutatum* Simmonds ex Simmods causes serious losses of strawberry fruits in Australia , South Africa , UK and California. Other species recorded on strawberries in the USA include *C. dematium C. fragaria* Brooks and *C . gloeosporioides*. It is difficult to distinguish between *C. acutatum* and *C. gloeosporioides*, however lesions on fruits are sunken and may be lined with a sticky mass of pink-colored spores. Black spot is first visible as a circular, slightly sunken, brown water soaked lesion, enlarging rapidly over a period of 2 or 3 days to involve most of the fruits. These are initially salmon- colored but after a period lesions become dark brown to almost black (Snowdon, 1990).

On the other hand, the causative agent was confirmed to be *C. acutatum* by PCR amplification using the time-specific primers TBCA and TB5 (Denoyes-Rothan, *et al.*, 2006, Porta-Puglia, and Mifsud, 2006, and Nam, *et al.*, 2008). Strawberry fruit rot with black and brown spots was observed in forcing culture in Saga prefecture, Japan. These symptoms were identified as *Colletotrichum acutatum* and *C. gloeosporioides* (Inada and Yamaguchi, 2006).

In Egypt, Anthracnose of strawberry caused by *C. acutatum* was recorded for the first time by Khafagi, 2006 and El-Marzoky, 2008, however, these reports did not rely on molecular analysis for accurate and reliable identification of the causal agent of anthracnose disease. This is thought to be the first reliable and accurate report, based on molecular identification, of *C. acutatum* and *C. gloeosporioides* causing anthracnose on strawberry in Egypt (Embaby, *et al*. 2010).

The aim of this work was focused to identify anthracnose pathogen(s) associated with diseased strawberry plants, root system, crown and fruit rots, based on the morphological cultural characteristics, pathogenicity test, Polymerase Chain Reaction technique (PCR) and susceptibility of various cultivars and effect of *C. acutatum* on fruit quality (physical, chemical characteristics and mineral contents).

Material and Methods

1- Isolation and identification of the causal agent:

Diseased samples of strawberry plants cv (s) Festival, Sweet Charlie, Tamar and Yael showing different symptoms Fig (1) i.e. stunting, wilting, root rotting, and death which described by **Arroyo, *et al*., (2008)** were uprooted from both two private.farms at Toukh, Kalubia and Kantara, Ismailia Governorates. Collected samples were prepared as follow: Roots and crowns were washed with tape water, cut into small pieces (1-2 cm), surface sterilized with 1% sodium hypochlorite solution for 2 min. then, washed several times with sterilized water and dried by using sterilized filter papers. Sterilized pieces of either roots or crowns were sown on autoclaved PDA medium supplemented with a 0.1% streptomycin sulphate to prevent bacterial contamination, then incubated at 26 ± 2°C for 5-7 days. All fungal colonies were transferred and purified on PDA medium (Thanab, *et al*., 2008).

Fig. 1: Healthy and infected crowns of strawberryTamar plants (natural infection).

Naturally rotted fruit samples of strawberry fruits from the same cultivars showing various types of rot symptoms i.e. discoloration, a circular black spot, slightly sunken, brown water soaked lesion, salmon- colored, turn dark brown to almost black were collected from two different localities i.e. Kalubia and Ismailia Governorates Fig (2). Samples were surface disinfested using 70% Ethyl alcohol for 2 min, washed with sterile water and dried at room temp., then cut into small pieces. Sterilized pieces were sown on sterilized PDA

medium amended with 0.1% streptomycin sulphate then incubated at 26 ± 2°C for 5 days. All fungal colonies were transferred and purified on PDA medium. Purified fungi were transferred on PDA slant medium and kept at 5°C for further studies.

2- Identification:

2.1- All isolated fungi were identified in Plant Pathology Dept., National Research Centre, El-Dokki, Egypt. Based on cultural and morphological characteristics, review of literature, and pathogenicity test in addition to PCR technique. *Colletotrichum* spp. was identified according to Frreman and Katan, (1997), Cannon, *et al.*, (2000), Frreman, *et al.*, (2002). and Schiller, *et al*., (2006).

Fig. 2: Anthracnose fruit rot symptoms onTamar strawberry (Natural infection) as a circular black spot, slightly sunken, brown water soaked lesion, enlarging rapidly of the fruit, these are initially salmon- colored.

2.2- PCR technique:

Isolation and purification of fungal DNA:

PCR amplification:

Species specific PCR was performed by using two primers as described by Sreenivasaprasad *et al.*, (1996) and Brown *et al.*, (1996). primers were CaInt2 (GGGGAAGCCTCTCGCGG) specific for *C. acutatum* and CgInt (GGCCTCCCGCCTCCGGGCGG) specific for *C. gloeosporioides*, each in combination with the conserved primer ITS4 (TCCTCCGCTTATTGATATGC).

3- Pathogenicity test and varietal reaction:

3.1– Source of the causal agent and transplants:

Two local species of fungal isolates which identified as *Colletotrichum acutatum* and *C. gloeosporioides* isolated from diseased strawberry plants were used in this experiment. Two different strawberry cultivars i.e. Tamar and Yael (which had been used as differential hosts) were kindly provided from Strawberry Improvement Centre, Fac. Agric. Ain-Shams Univ. which selected symptomless (healthy).

3.2 –Inoculums preparation:

The spore suspension from the two isolated was obtained as the methods of Kim, *et al.* (2008), Marta *et al.* (2008) and Shamim, *et al.* 2009).

3.3–Greenhouse-experiment:

Strawberry transplants cvs. Tamar and Yael (two months old) were planted in pots 25cm containing sterilized soil (one transplant/pot). Three replicates with six transplants per replicate were used for each treatment. Strawberry transplants were inoculated artificially by spraying a conidial suspension 1 X $(10)^6$ conidia/ml of *Colletotrichum acutatum* and *C. gloeosporioides*. Control plants were sprayed with sterile water (Marta, *et al.*, 2008, Kim, *et al.*, 2008 and Shamim, *et al.* 2009). All treated pots were covered by plastic sheet under 90% RH and 25-30°C. Susceptibility of the two different strawberry cultivars i.e. Tamar and Yael were studied according to Berta, *et al.*, (2008).
Disease incidence was estimated and recorded as follow:-

$$\text{Disease incidence \%} = \frac{\text{Total diseased plants}}{\text{Total inoculated plants}} \times 100$$

Regardless of appeared symptoms either as spot or lesions on leaves, stunted, wilted plants and mortality. *Colletotrichum acutatum* and *C. gloeosporioides* fungus were re-isolated again.

3.4– Laboratory studies:

Healthy strawberry leaves were surface sterilized with 1% sodium hypochlorite solution for 2 min. then, washed several times with sterilized water and dried by using sterilized filter papers. Sterilized strawberry leaves were inoculated artificially by spraying a conidial suspension 1 X $(10)^6$ conidia/ml of *Colletotrichum acutatum* and *C. gloeosporioides* fungus (Shamim, *et al.* 2009). Control treatment was inoculated with sterile water only. All treated leaves were covered by plastic sheet under a moist chamber with 90% RH and 25-30°c. Treated leaves were examined for any appeared symptoms which described by Arroyo, *et al.*, (2008). It depends on the appearance of spots or lesions on leaves.

Fruits of three different strawberry cultivars i.e. Sweet Charlie, Tamar and Yael were tested for their susceptibility to *Colletotrichum acutatum* and *C. gloeosporioides*. Healthy strawberry fruits at mature stage which obtained from Kalubia, fruits were surface disinfested using 70% Ethyl alcohol for 2 min, washed with sterile water then dried at room temp. and inoculated as mentioned before

$$\text{Infection \% was calculated as} = \frac{\text{Rotted fruits}}{\text{Total of fruits}} \times 100$$

The causal was re-isolated from inoculated rotted fruits.

4- Effect of C. acutatum on some physical, chemical characteristics and mineral contents of fruit:

Healthy strawberry fruits at mature stage were obtained from the private farm in Kalubia. were surface disinfested using 70% Ethyl alcohol for 2 min, washed with sterile water then dried at room temp and divided into two equal groups, the first was inoculated by fungi under test and the other was left as a control. Fruits were incubated at room temp. (25-30°C) under 90% RH. physical, chemical characteristics and mineral contents were determined. Fruit length (cm), fruit firmness (g/cm2). It was determined by using a Shatillon Penetrometer,total soluble solids (Brix°) samples of five fruits were randomly chosen to measure the percentage of soluble solids content using the hand refractmeter, Sugars contents were determined colormetrically by the method described by Schales and Schales (1945), pH value determine using pH meter, model 420A,vitamin C was determined in mg/100g fresh weight using 2, 6 dichloro – phenol indophenol by titration as the method mentioned in A.O.A.C. (2005),anthocyanin pigment was determined calorimetrically at 535 wave length according to De loose (1970).Loss and % reduction were calculated as follow:-

Loss = Healthy fruits – Infected fruits

$$\text{Reduction \%} = \frac{\text{Healthy fruits- Infected fruits}}{\text{Healthy fruits}} \times 100$$

As for mineral determination, fruit samples were taken at 120 days from planting and oven-dried at 70 C° until constant weight and ground to pass a 1 mm sieve then 0.1 g of the dry samples was taken and digested using a mixture of sulphuric acid (H_2SO_4 98 %) and hydrogen peroxide (H_2O_2 30 %) as described by Thomas *et al.* (1967).Total nitrogen was determined using Kjeldahl method as described by Page *et al.*, (1982). Phosphorus content was measured spectrophotometrically using the ascorbic acid method (AOAC, 2005). Potassium was measured by flame photometer as described by Page *et al.* (1982).Ca, Mg, Fe and Mn content was measured as mentioned in AOAC, (2005)

Results and Discussion

1. Percentage of total fungal count occurred with diseased strawberry plants:

Results in Table (1) show that Isolation of fungal pathogens associated with strawberry root system which collected from two different localities i.e. Kalubia and Ismailia in Egypt yielded 520 fungal isolates belonging to 150 isolates (equal 28.85%) from Kalubia location and 370 fungal isolates (equal 71.15%) from Ismailia location**.** Diseased samples of strawberry cultivars i.e. Festival, Sweet Charlie, Tamar and Yael showing different symptoms i.e. stunting, wilting, root rotting, and death indicate that, Yael cv. gave the highest percentage of total fungal count occurred which record 178 isolates (34.2%) followed by Tamar cv. which

recorded 161 isolates (31.0%). Festival was moderate which gave 110 isolates (equal 21.1%), while Sweet Charlie was the lowest which gave 71 fungal isolates 13.7%.

Table 1: Count and percentage of total fungi associate with diseased strawberry plants in Egypt. (2010 season).

		Cultivars								Total
		Festival		Sweet Charlie		Tamar		Yael		
Location		Crown	Root	Crown	Root	Crown	Root	Crown	Root	
Kalubia	*T. C	3	24	0	7	19	33	31	33	150
	%	0.57	4.62	0.0	1.35	3.65	6.35	5.96	6.35	28.85
Ismailia	T. C	38	45	36	28	61	48	44	70	370
	%	7.31	8.65	6.92	5.39	11.73	9.23	8.46	13.46	71.15
Total	T. C	41	69	36	35	80	81	75	103	520
		110		71		161		178		
	%	21.1		13.7		31.0		34.2		100.0

*T. C = Total fungal count

2. Percentage of fungal genera detected with diseased strawberry plants:

Data in Table (2a&b) show that, seven fungal genera were identified. These are *Colletotrichum, Fusarium, Macrophomina, Pestalotia, Rhizoctonia, Sclerotinia* and *Trichoderma* from both Kalubia and Ismailia regions. Also, data presented that, Kalubia samples exhibited lethal fungal count than Ismailia samples. Fusaria were the most frequently genus (belonging to *F. oxysporum*, *F. solani* and *Fusarium* spp.) in both Kalubia and Ismailia samples than other fungal genera. *Macrophomina phaseolina* was less of the total fungal count which gave 4 isolates equal 2.6% in Kalubia samples and 65 isolates of the total count with 23.2% in Ismailia samples. *Rhizoctonia solani* gave 58 isolates of total fungal count (15.7%) in Ismailia samples and 30 of total fungal count equal 20% in Kalubia samples. Also, the same table shows that, *Pestalotia longisetula* recorded 16 isolates of the total fungal count (10.7%) in Kalubia and 10 isolates of total fungal count (2.7%) with Ismailia samples. *Sclerotinia sclertiorum* gave 10 isolates of total fungal count (6.7%) and 17 isolates of total fungal count (4.6%) in both Kalubia and Ismailia samples respectively. The non pathogenic fungus *Trichoderma* sp. recorded 10 isolates of total count (6.7%) and 7 of total fungal count equal 1.9% with Kalubia and Ismailia samples respectively. Similar results were obtained by Michail *et al.,* (1980), Fahim *et al.,* (1998), Aref, (2005), Embaby, (2007a) and Embaby, *et al.,* (2008).who detected such fungal genera in strawberry plants.

On the other hand, *Colletotrichum* spp (the causal agent of strawberry anthracnose) was lower in Kalubia than Ismailia region. *Colletotrichum* spp. record 12 isolates of total fungal count equal 8% in Kalubia region compared with Ismailia region which reached to 65 isolates of total fungal count equal 17.6% respectively. The same results were obtained by Freeman and Katan (1997) and Garrido, *et al.* (2008).

Table 2a: Percentage of fungal genera occurred with diseased strawberry plants which collected from Toukh, Kalubia region . (2010 season)

Fungal isolates	Festival			Sweet-Charlie		Tamar		Yael		T.C	%
	Crown		Root	Crown	Root	Crown	Root	Crown	Root		
Colletotrichum spp.	T.c	0	0	0	0	2	2	6	2	12	
	%	0.0	0.0	0.0	0.0	1.3	1.3	4.0	1.3	8.0	
Fusarium oxysporum	T.c	2	3		1	2	2	4	2	16	
	%	1.3	2.0	0.0	0.7	1.3	1.3	2.6	1.3	10.7	
F. solani	T.c	1	7	0	2	3	16	2	7	38	
	%	0.7	4.7	0.0	1.3	2.0	10.7	1.3	4.7	25.3	
Fusarium spp.	T.c	0	11	0	3	0	0	0	0	14	
	%	0.0	7.3	0.0	2.0	0.0	0.0	0.0	0.0	9.3	
Macrophomina	T.c	0	3	0	1	0	0	0	0	4	
	%	0.0	2.0	0.0	0.7	0.0	0.0	0.0	0.0	2.6	
Pestalotia longisetula	T.c	0	0	0	0	2	4	3	7	16	
	%	0.0	0.0	0.0	0.0	1.3	2.7	2.0	4.7	10.7	
Rhizoctonia solani	T.c	0	0	0	0	9	6	8	7	30	
	%	0.0	0.0	0.0	0.0	6.0	4.0	5.3	4.7	20.0	28.8
Sclerotinia sp.	T.c	0	0	0	0	1	2	5	2	10	
	%	0.0	0.0	0.0	0.0	0.7	1.3	3.3	1.3	6.7	
Trichoderma sp.	T.c	0	0	0	0	0	1	3	6	10	
	%	0.0	0.0	0.0	0.0	0.0	0.7	2.0	4.0	6.7	
Total	T.c	3	24	0	7	19	33	31	33	150	
%	%	2.0	16.0	0.0	4.6	12.7	22.0	20.7	22.0	100.0	

*T. c= Total fungal count

Table 2b: Percentage of fungal genera occurred with diseased strawberry plants which collected from Kantara, Ismailia region. (2010 season)

Fungal isolates	T.C	Festival		Sweet Charlie		Tamar		Yael		Total	
		Crown	Root	Crown	Root	Crown	Root	Crown	Root	T.c	%
Colletotrichum spp.		0	0	0	0	12	9	25	19	65	
	%	0.0	0.0	0.0	0.0	3.2	2.4	6.6	5.1	17.6	
Fusarium oxysporum	T.C	1	0	2	0	1	0	1	0	5	
	%	0.3	0.0	0.5	0.0	0.3	0.0	0.3	0.0	1.4	
F. solani	T.C	12	13	9	12	12	11	6	8	83	
	%	3.2	3.5	2.4	3.2	3.2	3.0	1.6	2.2	22.4	
Fusarium spp.	T.C	4	11	6	5	4	6	0	3	39	
	%	1.0	3.0	1.6	1.4	1.0	1.6	0.0	0.8	10.5	
Macrophomina	T.C	20	11	17	8	23	7	0	0	86	
	%	5.4	3.0	4.6	2.2	6.2	1.9	0.0	0.0	23.2	
Pestalotia longisetula	T.C	1	2	0	0	1	1	2	3	10	
	%	0.3	0.5	0.0	0.0	0.3	0.3	0.5	0.8	2.7	
Rhizoctonia solani	T.C	0	3	2	1	6	8	10	28	58	
	%	0.0	0.8	0.5	0.3	1.6	2.2	2.7	7.6	15.7	71.2
Sclerotinia sp.	T.C	0	3	0	2	2	6	0	4	17	
	%	0.0	0.8	0.0	0.5	0.5	1.6	0.0	1.0	4.6	
Trichoderma sp.	T.C	0	2	0	0	0	0	0	5	7	
	%	0.0	0.5	0.0	0.0	0.0	0.0	0.0	1.4	1.9	
Total		38	45	36	28	61	48	44	70	370	
%		10.3	12.2	9.7	7.6	16.5	12.9	11.9	18.9	100.0	

*T. C = Total count

3. Percentage of total fungal count associated with strawberry rotted fruits:

Percentages of total fungal count were tabulated in Table (3). Rotted strawberry fruits which collected from the two different localities i.e. Kalubia and Ismailia Governorates cultivated with four strawberry cultivars i.e. Festival, Sweet Charlie, Tamar and Yael cvs. Yielded three hundreds of fungal isolates belonging to one hundred (equal 33.33%) from Kalubia samples and two hundred (equal 66.67%) of fungal isolates from Ismailia samples. Data in this table show that, five fungal genera were found to be associated with strawberry rotted fruits. These fungi were identified as *Aspergillus niger*, *Botrytis cinerea*, *Pestalotia longisetula*, *Rhizopus stolonifer* and *Colletotrichum* spp. Data also presented that, the occurrence of strawberry fruit rots in Ismailia location recorded higher percentage of total fungal count compared with Kalubia location. Also, Yael cv. was harbored higher frequency occurred of the total fungal count which gave 39% and 40% in both Kalubia and Ismailia respectively compared with other cvs. *Colletotrichum* spp. gave less percentage of total fungal count and occurred at 6.0% in both Kalubia and Ismailia samples respectively. Also data in the same table presented that, Sweet Charlie cv. recorded moderate percentage of total fungal count in Kalubia samples followed by Festival with 19.0% and Tamar cv. with 16%. Also, Sweet Charlie cv. gave moderate percentage of total fungal count in Ismailia samples which recorded 22.5% followed by Tamar cv. with 20% and Festival record 17.5%. *Rhizopus stolonifer* was the most frequent in Kalubia samples which recorded 38.0% of total fungal count followed by *Botrytis cinerea* with 33.0%. Moderate percentage of total fungal count was recorded with *Pestalotia longisetula* fungus with 17.0%. Both *A. niger* and *Colletotrichum* spp. were less and either recorded 6.o%. In Ismailia samples, *B. cinerea* gave higher percentage of total fungal and recorded 41.5%. *Pestalotia longisetula* was moderate which record 20.0% followed by *R. stolonifer* with 19.5% and *A. niger* with 13.0%.

Many authors reported that, *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Rhizopus stolonifer*, *Rhizoctonia solani*, *Phytophthora cactorum*, *Fusarium* spp., *Penicillium* spp. and *Sclerotinia sclerotiorum* are the most fungal isolates causing strawberry fruit rots in Egypt (Khafagi, 1982; Tadrous, 1991 and Tarek, 2004). In addition, *Pestalotia longisetula* was isolated and identified from strawberry rotted fruits (Embaby, 2007a & b). Morever, other strawberry fungal rotted fruits were isolated from different locations in all over the world i.e. *Aspergillus, Botrytis, Colletotrichum, Geotrichum, Mucor, Penicillium, Pestaliopsis, Phytophthora* and *Rhizopus stolonifer* were found infecting the crop (Fraire-Cordero, *et al.*, 2003 and Paivi, *et al*., 2008).

4. Identification of Colletotrichum spp.:

Two local species of *Colletotrichum* fungus i.e. *C. acutatum* and *C. gloeosporioides*, that were identified, based on the cultural and morphological characteristics and by Polymerase Chain Reaction technique (PCR). Morphological characterization identified all the tested isolates of *Colletotrichum* spp. as *C. acutatum* and *C. gloeosporioides*, which were further confirmed by species-specific polymerase chain reaction (PCR). Based on symptoms, cultural and morphological characteristics, pathogenicity test,and Polymerase Chain Reaction (PCR), the pathogen of anthracnose was identified and confirmed in Table (4). The same results were obtained by

Maymon, *et al*., (2009), they reported that, the *Colletotrichum* isolates were identified and characterized by classical morphological criteria and by various molecular methods.

Table 3: Total count and percentage of fungal frequency associated with strawberry rotted fruits. (2010 season).

Location	Cultivar	TC	*A*	*B*	*C*	*P*	*R*	Total
Kalubia	Festival	TC	2	10	0	4	3	19
		%	2.0	10	0.0	4.0	3.0	19.0
	Sweet Ch.	TC	0	10	0	6	10	26
		%	0.0	10.0	0.0	6.0	10.0	26.0
	Tamar	TC	3	5	1	2	5	16
		%	3.0	5.0	1.0	2.0	5.0	16.0
	Yael	TC	1	8	5	5	20	39
		%	1.0	8.0	5.0	5.0	20.0	39.0
	Total	TC	6	33	6	17	38	100
		%	6.0	33.0	6.0	17.0	38.0	100.0
Ismailia	Festival	TC	6	20	0	5	4	35
		%	3.0	10.0	0.0	2.5	2.0	17.5
	Sweet Ch.	TC	5	20	0	10	10	45
		%	2.5	10.0	0.0	5.0	5.0	22.5
	Tamar	TC	5	21	4	5	5	40
		%	2.5	10.5	2.0	2.5	2.5	20.0
	Yael	TC	10	22	8	20	20	80
		%	5.0	11.0	4.0	10.0	10.0	40.0
	Total	FC	26	83	12	40	39	200
		%	13.0	41.5	6.0	20.	19.5	100.0

TC= Total Fungal Count.
A= *Aspergillus niger.* B= *Botrytis cinerea.*
C= *Colletotrichum* spp. P= *Pestalotia longisetula.*
R= *Rhizopus stolonifer*

A: Morphological characterization:

Colletotrichum isolates produced colonies with beige to grey color on the top side and salmon-orange to pink color on the reverse side. Similar results were obtained by Zvezdomir, *et al.,* (2008). Bright orange conidial masses were formed mostly in the centre and occasionally at the edges of the colonies. The conidia were predominantly bursiform in shape with both ends tapered to a point. Also, data in Table (4) show that, *Colletotrichum* spp. produced numerous of conidial spores. The conidia had a single cell. A representative culture on PDA medium, which isolated from roots of wilted Tamar cv. plants possessed conidia which were hyaline, oblong with obtuse ends measuring 12.6 (11.8- 15.4) x 4.1(3.3- 5.1) micrometer as *C. acutatum* J.H. Simmonds (teleomorph *Glomerella acutata* J.C. Guerber & J.C. Correll). A representative culture on PDA medium, which isolated from roots of wilted Yael cv. plants possessed conidia which were cylindrical and attenuated at both ends, measuring 15.5 (14.3- 17.3) x 4.5(4.3- 5.0) micrometer as *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. [teleomorph *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk]. According to the above-mentioned morphological characteristics, all isolates in this study were similar and corresponded to the descriptions of *Colletotrichum* spp. These results are in agreement with those of Freeman and Katan, (1997), Cannon, *et al.,* (2000), Freeman, *et al.,* (2006), Schiller, *et al.,* (2006), Garrido, *et al.,* (2008) and Zvezdomir, *et al.,* (2008).who detected the two species of *Colletotrichum* in strawberry

Table 4: Cultural, morphological, pathological and molecular (PCR analyses) characterization of two local species of Colletotrichum.

Characters	*C. acutatum*	*C. gloeosporioides*
Culture	Forming a white aerial mycelium with ink spore masses, salmon color then become gray.	a white aerial mycelium then become dark to black .
Conidia	Are hyaline, cylindrical and attenuated at both ends.	Hyaline, oblong with obtuse ends.
Measuring conidia (size)	12.6 (11.8-15.4) X 4.1 (3.3-5.1) micrometer	15.5 (14.3-17.3) X4.5 (4.3-5).0micrometer
PCR amplification	With an amplified product 490 bp using primers ITS4 and *Ca*Int2.	A single amplified DNA of 450 bp using primers ITS4 and *Cg*Int.
Pathogenicity	Stunted and wilted plants. Necrotic lesions were observed on roots and crowns . Plants may die.	Wilted and stunted plants. Plants may die.

B- Species-specific primer analyses:

Species-specific PCR amplification in Fig (3) show that, *C. gloeosporioides* resulting in a single amplified DNA fragment of 450 bp using primers CgInt and ITS4. According to amplification products of genomic DNA from: *C. gloeosporioides* isolates from strawberry (lane 1, representative isolate CG-317 standard; lane 4, isolate 1). The same results were obtained by Freeman, *et al.,* (2006), and Zvezdomir, *et al.,* (2008). Also,in Fig (3)

Species-specific PCR amplification show that, *C. acutatum* with an amplified product of 490 bp using primers CaInt2 and ITS4. *C. acutatum* isolates from strawberry (lane 2, representative isolate TUT-149 standard; lane 3, isolate 4) and water control (lane 5). Lane M: DNA 1 kb marker ladder. Similar results were recorded by Ana, *et al*., (2008), Mymon, *et al.*, (2008) and Zvezdomir, *et al*., (2008).

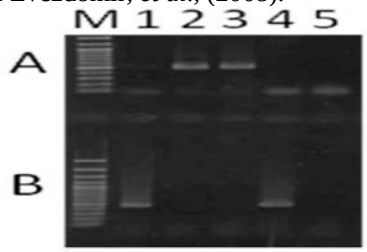

Fig. 3: Taxon-specific identification of *Colletotrichum acutatum* (A – primer CaInt2 in combination with primer ITS 4) and *C. gloeosporioides* (B - primer CgInt in combination with primer ITS 4) according to amplification products of genomic DNA from: *C. gloeosporioides* isolates from strawberry (lane 1, representative isolate CG-317 standard; lane 4) and *C. acutatum* isolates from strawberry (lane 2, representative isolate TUT-149 standard; lane 3) and water control (lane 5). Lane M: DNA 1 kb marker ladder.

5. Effect of Pathogenicity test on strawberry plants under greenhouse condition:

Data presented in Table (5) show that, both two local species i.e. *C. acutatum* and *C. gloeosporioides* were found to be pathogenic to strawberry plants either Tamar and Yael cv. Data in this table indicate that, *C. gloeosporioides* is more virulent than *C. acutatum* which recorded higher infection percent as well as percentage of mortality. Also, these data show that, increasing of infection percent as well as percentage of mortality with increasing the period after inoculation. After two weeks from inoculation, the plants began to exhibit wilt symptoms with the occurrence of severe symptoms being observed 4 weeks following inoculation. The *C. gloeosporioides* caused more severe infection of leaves reaching from 83% to 100% and mortality reaching from 67 to 83% than those of *C. acutatum* which reaching infected leaves from 33 to 58% and mortality of plants reaching from 50 – 83%, respectively. Similar results were recorded by Freeman, *et al*. (2006).

Typical anthracnose symptoms were observed in the inoculated plants on leaves, petioles and eventually on the developing flowers for both representative species i. e. black leaf spots, dark and sunken lesions also developed on the petioles and stolons, while control plants remained healthy Fig (4.a, b & c). Similar results were reported by Ana, *et al.,* (2008) and Arroyo, *et al.*,(2008). Also, tattered leaf lesions were appeared on the upper and lower leaves under moist chamber in laboratory Fig (5). Tissues with lesions began to rot and/or blight when lesions enlarged and coalesced 6 to 8 days after inoculation. Control leaves (untreated) had no symptoms Fig (5). A reddish brown, streaking or vascular color were occurs of crown wilted plants Fig (6). The same results were obtained by Denoyes-Rothan, *et al*., (2003), Arroyo, *et al.* (2008), Garrido, *et al*., (2008), and Shamim, *et al.* (2009).

Table 5: Effect of Pathogenicity test on nucleus and foundation strawberry plants cvs Tamar and Yael under greenhouse condition (2010 season)

Period week		Tamar cv.						Yael cv.					
		1-1			1-2			2-1			2-2		
		L	M	F	L	M	F	L	M	F	L	M	F
2	Cg	100.0	66.67	66.67	83.33	83.33	16.67	100.0	66.67	100.0	100.0	83.33	0.0
	Ca	50.0	50.0	00.00	33.33	50.0	0.0	33.33	33.33	50.0	33.33	50.00	100.0
4	Cg	100.0	66.67	66.67	83.33	50.0	16.67	100.0	83.33	100.0	100.0	83.33	0.0
	Ca	58.0	50.0	16.67	33.33	05.50	0.0	33.33	33.33	16.67	33.33	50.0	100.0

Cg = *C. gloeosporioides.* Ca = C. *acutatum*
1-1 = Tamar nucleus,plants 1–2 = Tamar foundation, plants
2-1 = Yael nucleus,plants 2-2 = Yael foundation, plants
L=Leaves % M=Mortality % F= infected fruits%

The same table presented that symptoms were reproduced by artificial inoculation of Tamar and Yael cvs. with the two representative species of *C. gloeosporioides* and *C. acutatum*, causes sudden wilt and collapse of the plants Fig (4 c). Plants may die. A reddish brown, streaking occurs of crown wilted plants Fig (6). These phenomenon's were confirmed by Zvezdomir, *et al.,* (2008).

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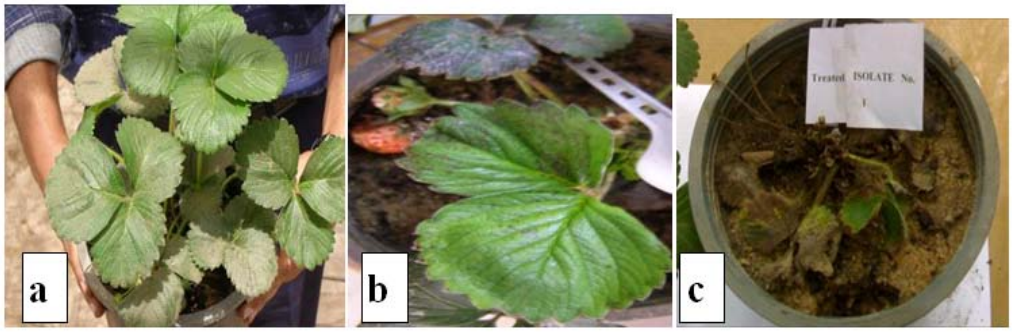

Fig. 4: Artificial inoculation of strawberry plants. Healthy (a) and infected plants (b): Leaf lesions were appeared. Sudden wilt and collapse of the plants (c).

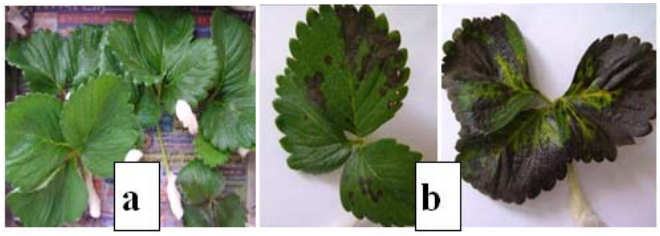

Fig. 5: Healthy (a) and infected leaves (b): A tattered leaf lesions were appeared on the upper and lower leaves under moist chamber in laboratory.

Fig. 6: Un-inoculated and inoculated crown of strawberry plants Tamar and Yael cvs. A reddish brown, streaking occurs of crown wilted plants.

6. Reactions of strawberry cultivars to Colletotrichum spp. infection:

Two strawberry cultivars i.e. Tamar and Yael were tested for their susceptibility to *Colletotrichum acutatum* and *C. gloeosporioides* fungus which inoculated by spraying a conidial (10)6 conidia/ml under a moist chamber. Data presented in Table (5) show that, Yael cv, was the most susceptible than Tamar cv. Also, data indicate that, *C. gloeosporioides* showed higher activity than *C. acutatum*. Both Tamar and Yael nucleus were the most susceptible to anthracnose infection compared with Tamar and Yael foundation plants respectively. Similar results were recorded by Freeman, *et al*., (2006) and Berta, *et al.*, (2008).

7. Effect of Pathogenicity test on strawberry fruits under greenhouse condition:

Data in Table (5) and Fig (7a &b). show that, both two local species of *Colletotrichum* were found to be pathogenic and causing typical anthracnose symptoms on fruits, petioles and flowers. Artificial inoculation of strawberry fruits caused a circular spot, slightly sunken, brown water soaked lesion, enlarging rapidly of the fruit, these are initially salmon- colored but after a period of host weather, turn dark brown to almost black. Also, data indicated that, *C. gloeosporioides* was more virulence than *C. acutatum* on Tamar and Yael cvs. Infection was increased by increasing incubation period. These results are in agreement with those reported by Denoyes-Rothan, *et al.*, (2006), Porta-Puglia, and Mifsud, (2006), Berta, *et al.*, (2008) and Nam, *et al*., (2008). The highest lesions on fruits were caused by *C. acutatum* but on petioles and crowns. On the other hand, Yael cv. was highly susceptible than Tamar cv.

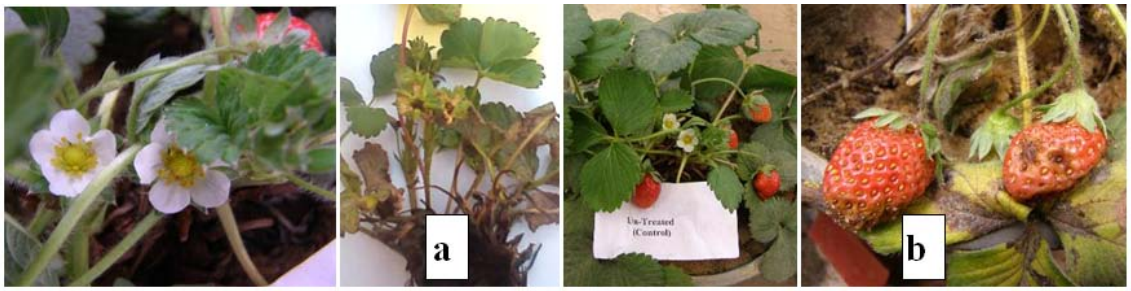

Fig. 7: Artificial infection symptoms.

(7.a): The of infected flowers turns brown to black compared with untreated.
(7.b): Typical symptoms were appeared on strawberry fruits**,** as a circular spot, slightly sunken, brown water soaked lesion, enlarging rapidly of the fruit, these are initially salmon- colored but after a period of host weather, turn dark brown to almost black compared with untreated (control).

8. Reactions of strawberry cultivars to Colletotrichum spp. infection in laboratory:

Three strawberry fruit cultivars i.e. Sweet Charlie, Tamar and Yael were tested for their susceptibility to *Colletotrichum acutatum* and *C. gloeosporioides* fungus. Data in Table (6) indicate that, both two species i.e. *C. acutatum* and *C. gloeosporioides* were found to be pathogenic to fruits in all tested cultivars and causing typical anthracnose symptoms. Yael cv, was the most susceptible than the others. Also, *C. gloeosporioides* gave higher infection percent with all tested cultivars i.e. Sweet Charlie, Tamar and Yael which recorded 60, 63 and 80% respectively, while *C. acutatum* gave less infection and recorded 30, 35 and 40% in fruits with the same cultivars respectively.

Lerceteau-Kohler, *et al.,* (2004) reported that, Capitola was resistant to the *C. acutatum*-pathogenicity group 1 (pg1) and to *P. cactorum* while 'CF1116' was susceptible to *C. acutatum* and moderately susceptible to *P. cactorum*. Also, 'Sweet Charlie' continues to be grown because of its resistance to diseases caused by *Colletotrichum acutatum* in west central Florida, this cultivar is field-immune to anthracnose fruit rot and resistant to *Colletotrichum* crown and root necrosis (Chandler, 2006). Cultivar Camarosa was susceptible to anthracnose, whereas 'Medina' could be considered moderately susceptible. Susceptibility of four wild species was similar to Camarosa. *F. mosachata* and *F. virginiana* species were more resistant to anthracnose crown rot than Camarosa (Simson *et al.*, 2006). Sweet Charlie, Carmine, and Earlibrite were the most resistant cultivars tested; Festival was intermediate in susceptibility; and Camarosa and Treasure were highly susceptible (Chandler, *et al.,* 2006). Chandler, Elsanta and Valeta were more susceptible than Dover and Seascape (Simpson, *et al.* 2006). *Colletotrichum gloeosporioides* isolated from strawberry were tested for virulence on strawberry cultivars in field experiments for three seasons. Thus, resistance to *C. gloeosporioides* appears to be nonspecific. The cultivar Treasure was more resistant to crown rot caused by either species than any other cultivar tested (MacKenzie, *et al.*, 2006). Determining the susceptibility of seven strawberry fruit cultivars to two fungal rot pathogens i.e. *B. cinerea* and *C. acutatum* resulted that, significant differences of severity between strawberry fruit cultivars for gray mold rot and fruit rot caused by *C. acutatum.* Gaviota was more susceptible to *B. cinerea* (58% of severity) but Ventana was more susceptible to *C. acutatum* (62% of severity)as found by Garrido, *et al*., (2008).

Table 6: Pathogenicity test on strawberry fruits which data was recorded after 5 days of inoculation. (compined analysis for 2011 and 2012 seasons).

Fungal species	Sweet Charlie	Tamar	Yael
C. gloeosporioides	60.0	63.16	80.0
C. acutatum	30.0	35.0	40.00

9. Effect of Colletotrichum infection on vegetative growth of strawberry plants:

Data in Table (7) show that, Colletotrichum infection decreased significantly the percentage of plant survival from 93.90 to 83.57%, number of leaves from 13.90 to 8.90, leaf area from 34.00 to 23.97 cm^2, plant dry weight from 16.83 to 11.70 g and decreased the plant height from 27.13 to 20.10cm.

Table 7: Effect of Colletotrichum infection on some growth characteristics of strawberry plants. (compined analysis for 2011 and 2012 seasons).

Character	Healthy	Infected
Plant survival %	93.90a	83.57b
No. of leaves	13.90a	8.90b
Leaf area (cm^2)	34.00a	23.97b
Plant height (cm)	27.130a	20.10b
Plant dry weight (g)	16.830a	11.70b

Values in the same rows followed by the same letter(s) do not significantly differ from each other according to Duncan's multiple range tests at 5% level.

10. Effect of C. acutatum on yield losses (fruit quantity and quality) of strawberry:

10.1-Physical characteristics:

Data in Table (8) indicate that, inoculated strawberry fruits (with *C. acutatum*) had significant lower diameter compared with healthy once (un-inoculated control). The average weight (g) of inoculated fruit was 10.25g, while the average weight of healthy fruit was 13.21g with 22.41% of reduction. The lowest marketable fruit length was. 2.75% cm and the highest was significantly. 3.45cm with 20.29% reduction in treated and un-treated fruits respectively. Also, the fruit diameter decreased significantly from 2.35 to 1.95cm in healthy and infected fruits respectively with 17.02% reduction. Fruit firmness was reduced significantly from 132.12 to 125.75 g/cm^2 with 6.37 losses and 4.82% reduction. Also data show, decreased fruit volume from 16.75 to 15.82 cm^3 in inoculated and non-inoculated (control) respectively with 5.55% reduction. Similar results were obtained by Timudo-Torrevilla, *et al.*, (2005), Embaby, (2007 b), Agulheiro-Santos, and Barreto, (2008) and Perez, *et al*., (2008).

Table 8: Effect of *Colletotrichium acutatum* on some physical characteristics in strawberry rotted fruits (Tamar cv.) after 7 days from inoculation(compined analysis for 2011 and 2012 seasons).

Parameters	Healthy	Infected	Loss*	% Reduction
Fruit weight (g)	13.21a	10.25b	2.96	22.41
Fruit length (cm)	3.45a	2.75b	0.70	20.29
Fruit diameter (cm)	2.35a	1.95b	0.40	17.02
Fruit firmness(g/cm^2)	132.12a	125.75b	6.37	4.82
Fruit volume (cm^3)	16.75a	15.82a	0.93	5.55

Values in the same rows followed by the same letter(s) do not significantly differ from each other according to Duncan's multiple range tests at 5% level.
* Loss = Healthy - Infected.

10.2- Chemical characteristics:

Data in Table (9) presented that, inoculated fruits by *C. acutatum* had lower significantly TSS (4.38%) compared with healthy (un-inoculated) fruits (12.50%) with 8.12 loss and 64.96% reduction. On the other hand, higher significant values of juice pH 3.75 were recorded with inoculated fruits compared with un-inoculated (control) fruits which record 3.25 with 13.33% reduction. Also, significant decline in sugar contents from 5.45 to 4.95 mg/100g in healthy and inoculated fruits respectively with 9.17% reduction. Total anthocyanins showed non significant decrement in infected fruits than non infected ones whereas ascorbic acid was decreased significantly from 45.57mgin control (healthy) fruit to 40.85mg/100g with 4.72mg/100g loss and 10.36% reduction in inoculated fruits. Similar results were obtained by Lerceteau-Kohler, *et al*., (2004), Timudo-Torrevilla, *et al.*, (2005), Embaby, (2007 b), Agulheiro, and Barreto, (2008) and Perez, *et al*., (2008).

Table 9: Effect of *Colletotrichium acutatum* on some chemical characteristics in strawberry rotted fruits (Tamar cv.) after 7 days from inoculation. (compined analysis for 2011 and 2012 seasons)

Parameters	Healthy	Infected	Loss*	% Reduction
T.S.S (%)	12.50a	4.38b	8.12	64.96
PH of Juice	3.25b	3.75a	0.50	13.33
Total sugars (mg/100g)	5.45a	4.95b	0.50	9.17
Anthocyanin (mg / l00 g)	75.25a	70.12a	5.13	6.82
Ascorbic acid (mg/l00 g)	45.57a	40.85b	4.72	10.36

Values in the same rows followed by the same letter(s) do not significantly differ from each other according to Duncan's multiple range tests at 5% level.
* Loss = Healthy - Infected.

10.3- Mineral contents:

Data recorded in Table (10). show that, infected fruits with C. *acutatum* gave significantly lower values in nitrogen, phosphorus, iron and manganese on the other hand,no significant differences were detected between healthy and infected fruits in their contents of potassium, calsium and magnisium and manganese . Similar results were obtained by Lerceteau-Kohler, *et al.,* (2004), Timudo-Torrevilla, *et al.,* (2005), Embaby, (2007 b), Agulheiro-Santos, & Barreto, (2008) and Perez, *et al.*, (2008).

Table 10: Effect of *Colletotrichium acutatum* on some mineral content in strawberry rotted fruits Tamar cv. after 7 days from inoculation(compined analysis for 2011 and 2012 seasons).

Mineral analytically	Healthy	Infected	Loss*	% Reduction
N . (mg)	0.82a	0.70b	0.12	14.63
P . (mg)	0.28a	0.18b	0.10	35.71
K . (mg)	0.42a	0.81a	0.39	11.96
Ca . (mg)	0.31a	0.25a	0.06	19.36
Mg . (mg)	0.15a	0.13a	0.02	13.33
Fe . (P P M)	110.21a	102.75b	7.46	7.22
Mn (P P M)	75.25a	71.35b	3.90	5.18

Values in the same rows followed by the same letter(s) do not significantly differ from each other according to Duncan's multiple range tests at 5% level.
*Loss = Healthy - Infected.

It could be concluded that PCR technique can be used to detect *Colletotrichum* spp. the causal agent of anthracnose disease in strawberry. Anthracnose disease (*Colletotrichum* spp.) causes severe losses in strawberry production. Infected fruits had lower values of most physical and chemical characteristics in addition mineral content(s) than healthy ones. All healthy fruits showed better and gave good quality, whereas all infected fruits presented poor quality.

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