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Molecular Technology Confirms Identification of Cantaloupe Anthracnose Caused by *Colletotrichum* sp.

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Anthracnose symptoms on cantaloupe plants were observed and collected during season of 2016 in Egypt. Because it is commonly known of its attacks on *Cucurbitaceae*, host range was studied. Seedling symptom test was followed for the purpose of host range study. While reactions of other non-cucurbit crops were evaluated for their susceptibility under greenhouse conditions. Host range studies resulted in infection ranged between 10 – 30%. Reaction of non-cucurbit crops proved variability in their susceptibility to current pathogen. Isolation and purification of the associated fungi were carried out and morphologically identified. The fungus was purified and morphologically identified as *C. lagenarium*. Since morphological characterization was sometimes misleading. PCR was implemented to confirm identification. The implemented PCR technique showed similarity of 99% and 100% of *C. chlorophyti* when ITS1 and ITS4 were amplified, respectively. Moreover, chemical control of this disease was evaluated. Soil drench efficiently reduced the disease incidence up to 73.9% using the fungicide Uniform 39%SE at 650cm3/fed. It could be concluded that host specificity of *Colletotrihcum* species was imperfect, using the polymerase chain reaction (PCR) along with morphological characters in identifying members of the genus *Colletotrichum* has become necessary and chemical treatments may be considered as a mean of controlling anthracnose in cucurbits. This finding, in addition to results of the host range, could be considered when conducting an anthracnose-resistance breeding program.

Keywords: *Anthracnose, Colletotrichum chlorophyti, C. orbiculare, host range, morphology, PCR and, seed treatment.*

Colletotrichum orbiculare (Berk.&Mont.) Arx is often known by its synonymized scientific name *C. lagenarium*, the asexual (anamorph) stage of the pathogen, while its sexual stage (telemorph) is *Glomerella lagenarium* (Walker *et al*., 1991). The pathogen is commonly able to attack members of Cucurbitaceae and to cause damages on certain crops including cucumber (*Cucumis sativu*s L.), pumpkin (*Cucurbita moschata* L.), watermelon (*Citrullus vulgaris* L.), cantaloupe (*Cucumis melon* var. *cantaloupensis* L.) and melon (*Cucumis melo* L.).

Colletotrichum orbiculare is widely distributed throughout the world and it had been reported from numerous countries in Asia, Africa, North America, South America, Central America, Europe and Oceania (Farr *et al*., 1989 and Koike *et al*., 1991). The fungus was reported on more than 40 plant host species worldwide (Leben and Daft, 1968; Monroe *et al*., 1997 and Farr and Rossman, 2013).

Symptoms were typical with lesions of circular to elongate dark brown on stems of diseased plants mostly near the surface of the ground, leaves, petioles and fruits (Anon., 2008; Egel, 2010 and Anon., 2014).

Typical anthracnose symptoms in some cantaloupe fields grown under sprinkling irrigation were observed for the first time in Egypt. Isolation and purification from the collected diseased plants were carried out and morphologically identified as *C. lagenarium* (El-Wakil and El-Zefzaf, 2001). Many workers in the field of taxonomy concluded that morphological characters alone were not reliable criteria for Colletotrichum identification and have failed to resolve the phylogenetic relationships among the species (Weir *et al*., 2012; Chen *et al*., 2015 and Sangpueak *et al*., 2017). According to the Agricultural Pesticide Committee (Anon., 2017), no fungicides were recommended in Egypt until 2018 as a foliar application to control anthracnose on cantaloupe because it is commonly edible fresh in Egyptian diets during the summer season and also because of restrictions related to exportation of such an important Agro-commodity.

The objectives of the current investigation were using PCR to confirm identification of the anthracnose isolated form cantaloupe. Also, studying host range of *C. orbiculare* within *Cucurbitaceae* or among non-cucurbit crops, and to throw some light on soil fungicidal drench to control anthracnose disease in cantaloupe.

Materials and Methods

In late summer, during September and October (2016), a survey of cantaloupe fields was conducted in El-Bostan, Beheira Governorate, Egypt looking for anthracnose infections. Anthracnose-like symptoms on cantaloupe plants (*Cucumis melo* L. var. *cantaloupensis* Naud.) including circular spots on leaves, leaf dryness, tiny black, saucer-shaped with setae were collected whenever observed by hand lens. Further studies and investigations were subsequently carried out.

Isolation and purification:

One cm segments of cantaloupe stems were surface-sterilized in 2.5% NaOCl for 3 min., washed for several times with sterilized distilled water, dried between sterile filter paper then placed on PDA medium and incubated at 25^{o}C with daily observation. Developed fruiting bodies were then picked off using a fine sterile glass needle and a stereoscopic microscope to transfer it into PDA slants and incubated for 10 days, then kept in refrigerator for further work.

Cultural characteristics:

Eight Petri dishes containing PDA medium were inoculated with single conidia and incubated at 20^{o}C ± 2 for two weeks under 12 h of alternating cycles of near ultraviolet light (NUV) and complete darkness. Colony features; mycelium characteristics, reproductive structures and overall colony appearance were described.

Host range:

Seedling symptom test developed by Khare *et al*. (1977) was followed to study the reaction of cucurbit crops to the isolated *Colletotrichum* sp., where eight

cucurbits crops *i.e.,* cucumber, melon, watermelon, squash, pumpkin, luffa, snake melon and cantaloupe were tested. Twenty seeds of each crop were individually sown in 16 mm diam. tubes containing water agar slant inoculated with 5 mm disk 7 days old bearing the fungal growth before sowing. Percentage of diseased plants was recorded after 20 days from seeding and calculated as mentioned below.

Reaction of some susceptible non-cucurbit crops to other *Colletotrichum* species was also carried out under greenhouse conditions. Soybean, chickpea, cotton, sugar beet, tomato, pea, bean, pepper, cowpea and the ornamental plant *Hibiscus cannabinus* were used in current experiment Sterilized pots of 30-cm diam. containing sterilized soil were infested with 0.4% (w:w) of 15-days old medium composed of fungal pathogen mixed with sand and sorghum, then watered every two days for a week to assist pathogen establishment. Un-infested soil was used as control. Surface sterilized seeds were washed four times in sterilized water then dried between two sterilized filter papers. Ten seeds of each crop were sown per pot and 4 replicates were used per each. Infection percentage was recorded and calculated after 30 days from seeding using the following formula:

$$\% \text{ Infected plants} = \frac{T - H}{T} \times 100$$

Where; T: total number of seedlings and H: number of healthy seedlings

Molecular identification:

Pure cultures of a single-spore were grown on PDA medium for 7 days at 25°C. The mycelia were scraped off with a scalpel from the plate and put into liquid nitrogen. The mycelia were subsequently ground to a fine powder and genomic DNA was extracted using the commercial DNeasy Plant Mini Kit™ (Qiagen, Germantown, MD, USA) following the manufacturer's guideline. *Colletotrichum* isolate was identified to the species level using sequencing data from the internal transcribed spacer (ITS) region. Amplification of ITS region was performed using PCR with primer ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and primer ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The PCR was prepared with 25 µl mixture final volume. Each reaction contained 0.2 µl Taq DNA polymerase (5U/ µl Promega), 5 µl of 10x buffer, 1 µl of 10 mm dNTPs, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl each of forward and reverse primers, 1 µl of genomic DNA (10 ng/µl) and adjusted to final volume by sterile distilled water. The amplification was done in a thermal cycler Techne, Touchgene Gradient, FTGRAD2D, 50-60 HZ starting with initial denaturation at 95°C for 5 min., followed by 35 cycles of denaturation step a 95°C 20 sec., annealing step at 55°C for 30 sec. and extension step at 72°C for 1 min., and finally extension at 72°C for 5 min. (Kuan *et al*., 2010). Gel electrophoresis was performed using aliquots of 10 µl of the PCR product on 1% agarose gels (w/v) running with 1x TBE buffer at 100 V for 25 min. The gel was post stained with 6x loading buffer (Gene DireX's Novel Juice, USA) and visualized under UV light. PCR was sequenced in forward and reverse orientation using the same primers for amplification. Purification of PCR products and cycle sequencing reactions were performed in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystem) at Inst. Biotechnol. for Postgraduate Studies & Res., Suez Canal Univ., Egypt. AB1

Trace files were analyzed using the sequence alignment editor and analysis. BioEdit and consensus sequence were adjusted to the BLST analysis on NCB1.

Effect of soil drench on the infection of cantaloupe by C. orbiculare under greenhouse conditions:

The effect of five different fungicides, *i.e.* Tachichem 30%SL, Uniform 39% SE, Combenix 70% WP, Defender 11.1% SS and Revanok 50% SL applied as soil drench against the infection of *C. orbiculare* was evaluated.

Cantaloupe seeds of Ideal cv. Were used in this experiment. Planting was carried out in infested soil as previously mentioned, 10 seeds were sown per pot and 4 replicates were used per treatment. Watered with fungicidal solutions only once according to the doses presented in Table 3 and then irrigated with tap water when needed. Un-infested soil was used as control treatment. The healthy looking survived plants were counted. Percentage of efficacy was recorded after 40 days and calculated as follows:

$$\% \text{ Efficacy} = \frac{C - M}{C} \times 100$$

Where; C: mean of control treatment and M: mean of fungicidal treatment

Results

Occurrence of C. orbiculare and disease symptoms:

Typical symptoms of anthracnose were observed in some cantaloupe fields grown under sprinkling irrigation in late growing season of 2016. Isolation and purification from the collected diseased plants were carried out and the obtained isolates were morphologically identified as *C. lagenarium* (= *orbiculare*) (Berk.&Mont.)Arx.

Symptoms appeared on all aerial plant parts, especially on crown area as brown lesions (Fig. 1: a&b). On the dried infected parts, black fruiting bodies were scatered (acervuli) with minute black setae that can be easily seen with the aid of hand lens (Fig. 1:c).

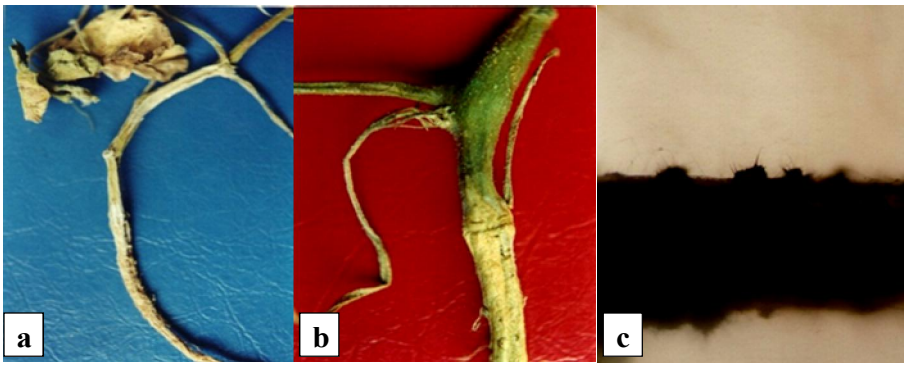

Fig. 1. Anthracnose symptoms as collected from cantaloupe plants: a&b: brown lesions and dryness as appeared on crown and aerial parts on diseased cantaloupe plants and (b) dried infected parts covered with black fruiting bodies or acervuli and c: Acervuli sparsely or thickly covered with minute black spines (setae).

Isolation and morphological characterization:

One isolate of *Colletotrichum* sp. was isolated from diseased cantaloupe plant parts using PDA medium. Colonies are dark grey to black, with paler margin (Fig. 2:a). Sclerotia abundant, acervuli single or in groups, setae are numerous blackish brown to black on color, longer than the conidial mass (Fig.2:b). One cell hyaline conidia formed in salmon pink masses straight or slightly curved pointed in both ends, 10-15 x 4.5-6 µ (Fig.2:c).

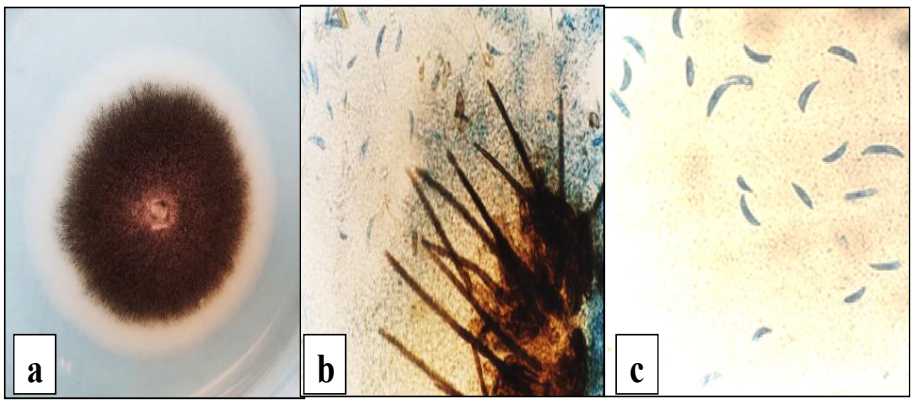

Fig. (2): characters of *C. lagenarium* (= *orbiculare*):
a: dark grey culture on PDA with paler margins.
b: a portion of mature acervulus with numerous brown blackish setae longer than the conidial mass (200X).
c: one hyaline cell conidia in salmon pink masses, straight or slightly curved pointed in both ends (750X).

Host range:

Data in Table 1 show that infection of 30% was recorded on artificially inoculated seedlings of cucumber, melon and cantaloupe. While, pumpkin, luffa and snake melon showed the least (10%) susceptibility to *C. orbiculare*. Squash and watermelon showed moderate infection (20%). Besides cucurbits, data in Table 2 show a total of ten non-cucurbit crops, among of which four field crops, five vegetables and one ornamental crop. Non-cucurbits used in the current experiment reacted differently against *C. orbiculare* when planted in artificially infested soil under greenhouse conditions.

Table 1. Susceptibility of come cucurbit crops to *C. orbiculare* using seedling symptoms test

Crop		Infection (%)
Common name	**Scientific name**	
Cucumber	*Cucumis sativus*	30
Melon	*Cucumis melo*	30
Watermelon	*Citrullus lanatus*	20
Squash	*Cucurbita pepo*	20
Pumpkin	*Cucurbita moschata*	10
Luffa	*Luffa cylindrica*	10
Snake melon	*Cucumis melo reticulatus*	10
Cantaloupe	*Cucumis melo var. cantaloupensis*	30

Regardless plant families, there were five crops showed no visible infection (0.0%) *i.e.* sugar beet, tomato, pea, pepper and hibiscus. On the other hand, soybean and cotton had almost similar infection levels, 17.8% and 17.7%, respectively. Chickpea, cowpea and bean showed variable reactions to the tested pathogen with records of 15, 10 and 7.5% infection, respectively (Table 2).

Table 2. Susceptibility of some non-cucurbit crops to *C. orbiculare*

Crop		Infection
Common name	Scientific name	(%)
Soybean	*Glycine max*	17.8
Chickpea	*Cicer arietinum*	15.0
Cotton	*Gossypyium barbadense*	17.7
Sugar beet	*Beta vulgaris*	0.0
Tomato	*Lycopersicon esculentum*	0.0
Peas	*Pisum sativum*	0.0
Bean	*Phaseolus vulgaris*	7.5
Pepper	*Capsicum annuum*	0.0
Cowpea	*Vigna unguiculata*	10.0
Hibiscus rosa	*Hibiscus cannabinus*	0.0

Molecular identification:

DNA of *Colletotrichum* isolate was successfully amplified and sequenced for ITS rDNA region. A fragment of approximately 547bp was amplified using ITS1 and ITS4 set in the tested isolate and no amplification of *Colletotrichum* isolate when compared to those of *Colletotrichum* accessions in the GenBank database, showed that the isolate had 99% and 100% similarity to *C. chlorophyti* accession

number MG543788.1 and MI1290362.1, respectively. The ITS rDNA region sequence of *C. chlorophyti* isolate generated in this study was deposited in GenBank had the accession number of MH664964. Plates 1 and 2 illustrate DNA analysis.

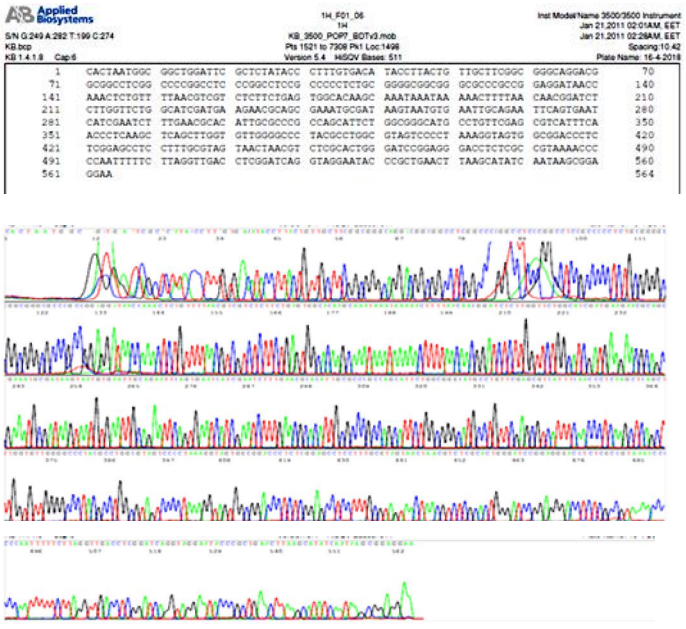

Plate (1)

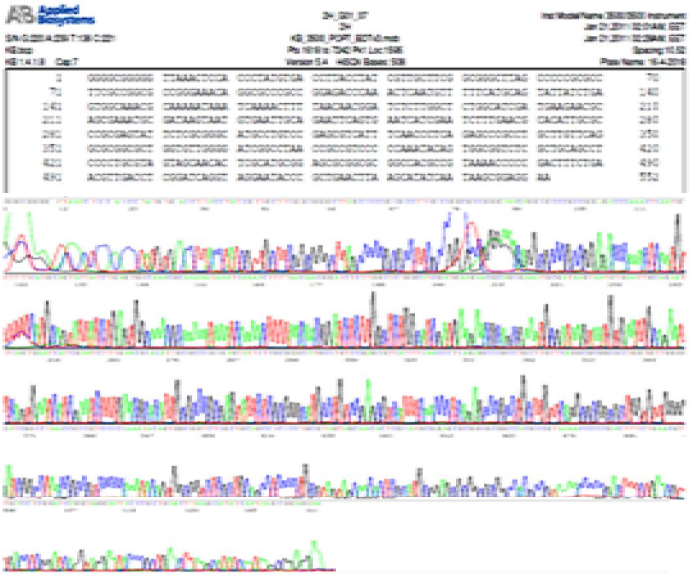

Plate (2)

Effect of soil drench treatments on the infection by cantaloupe C. orbiculare under greenhouse conditions:

The effect of five different fungicides *i.e.,* Tachichem 30%SL (hemexazole), Uniforme 39% SE (azoxystrobin + metalaxyl M), Combenix 70% WP (thiophanate-methyl), Defender 11.1% SS (copper sulfate anhydrous), and Revanok 50%SL (β-hydroxyquinoline sulfate) was tested as soil drench treatments once after sowing against the infection by *C. orbiculare*.

Data in Table 3 indicate that Uniforme 39% SE gave the highest suppression of anthracnose with 73.9% efficacy. Tachichem 30% SL and Combenix 70% WP gave disease control of 69.6% and 65.2% efficacy, respectively. Defender 11.1% SS and Revanok 50%SL were similar in their effect on the disease.

Table 3. Effect of five fungicides as soil drench on cantaloupe anthracnose caused by *C. orbiculare* under greenhouse conditions

Trade name	Nomenclature common name	Dose	Infection %	Efficacy %
Tachichem 30%SL	Hymexazole	1.5 cm^3/L	17.5	69.6
Uniform 39%SE	Azoxystrobin-metalaxyl M	650cm^3/fed.	15.0	73.9
Combenix 70%WP	Thiophanate-methyl	1 gm/L	20.0	65.2
Defender 11.1%SS	Copper sulfate abhydrous	2.5gm/L	22.5	60.9
Revanok 50%SL	β-hydroxyquinolin sulfate	1cm3/L	22.5	60.9
Control (without fungicides)			57.5	

D i s c u s s i o n

The cantaloupe anthracnose fungus was identified as *Colletotrichum lagenarium* (= *orbiculare*) (vonArx, 1957). Nikandrow *et al.* (1990) stated that anthracnose was considered the most destructive disease for a long period of time in most areas cultivating cucurbits. Anthracnose was relatively common in warm, humid regions worldwide; while in the USA, it was destructive in the south, southeast, northeast and Midwest regions where field losses were up to 60% (Wasilwa *et al.*, 1993). The species of *C. orbiculare* was considered to cause a complex disease appeared to be restricted to specific herbaceous host species/genera in four plant families, *Asteraceae, Cucurbitaceae, Fabaceae* and *Malvaceae*. *C. orbiculare* attacks species of several genera in the *Cucurbitaceae* (Damm *et al.*, 2013).

Current results confirmed that *C. orbiculare* could infect several non-cucurbit hosts. Meantime, it was pointed out that the genus *Colletotrichum* includes a number of plant pathogens of major importance, causing diseases of a wide variety of plants such as coffee berries, cereals including maize, sugar cane, sorghum, strawberry,

mango and banana (Cannon *et al.*, 2012). It was also stated that their understanding of the extent that *Colletotrihcum* species exhibits host specificity was imperfect. That was emphasized referring to a number of factors, including incomplete sampling, restriction of data largely to populations affecting crop or ornamental plants, and poor knowledge of pathogenic effects. Current results could be considered as additional evidence to that the genus *Colletotrichum* needs more research on the field of taxonomy and molecular characterization.

Traditionally, identification of *Colletotrichum* species has been based on morphological characters, such as size and shape of conidia and appressoria, existence of setae, the host origin, the teleomorph state, and cultural characteristics (Than *et al*., 2008; Peng *et al.*, 2012 and Peng *et al.,* 2013). In Egypt, El-Wakil and El-Zefzaf (2001) used the abovementioned parameters to identify current pathogen as *C. lagenarium*. On the contrast, Weir *et al.* (2012) and Chen *et al.* (2015) stated that the morphological characters alone are not reliable criteria for *Colletotrichum* identification and have failed to resolve the phylogenetic relationships among the species. Similar observations were found for identification of *Colletotrichum* species associated with cassava anthracnose (Sangpueak *et al.*, 2017). In the current study, it had been decided to confirm identification of the isolated fungus based on ITS sequence. Identification carried out through this investigation corrected the previous identification of the pathogen to another species called *C. chlorophyti*, that was according to its high similarity with those two isolates accessioned in the GenBank database.

In relation to chemical control of watermelon anthracnose which was considered as a compound interest disease and it could be effectively controlled by thiophanstate for as long as 20 days after application but it did not eradicate the pathogen from established infection. Generally, chemical treatment could be more effective if it was applied as early as the disease symptoms appeared (Amin and Ullasa, 1981).

Although results obtained in the current study may be considered as important findings for researchers, plant pathologists and taxonomists, meantime there are some related limitations. For instance, purity of DNA extracted from the pathogen should be in an excellent quality (Chen *et al.*, 2015). Other limitations might relate to facilities, expenses and accuracy.

Host specificity of *Colletotrihcum* species was imperfect, sothat advanced technologies such as polymerase chain reaction (PCR) besides morphological characters in identifying members of the genus *Colletotrichum* has become necessary. It had been proven that *C. orbiculare* may infect other plant hosts than cantaloupe. Soil drench may be considered as a safe means of controlling anthracnose in cucurbit crops.

These results afford a confirmed identification of the cause of the cantaloupe anthracnose. This finding in addition to results of the host range could be considered when conducting an anthracnose-resistance breeding program.

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استخدام التكنولوجيا الجزيئية لتأكيد تعريف أنثراكنوز الكنتالوب المتسبب عن نوع من جنس الكوليتوتريكم

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تم جمع نباتات الكنتالوب التي شوهدت فى موسم ٢٠١٦ ويظهر عليها أعراض مرض الأنثراكنوز من منطقة البستان بمحافظة البحيرة فى مصر. وبما أن المرض معروف بقدرته على مهاجمة نباتات العائلة القرعية فقد تم دراسة المدى العوائلى للمسبب الذى أحدث نسب إصابة تراوحت بين ١٠ – ٣٠% ، بالإضافة لتقييم بعض العوائل النباتية غير القرعيات من حيث قابليتها للإصابة فقد تم تسجيل اختلافات فى مدى قابليتها للإصابة من متحمل ومتوسط وقابل للإصابة. ولتأكيد التعريف الذى تم مسبقا باستخدام الصفات المورفولوجية على أن المسبب هو الفطر كوليتوتريكم لاجناريوم (= أوربيكيولير) ، فقد تم إعادة التعريف بتطبيق طريقة متطورة حيث استخدمت تقنية تفاعل البلمرة المتسلسل PCR التى أوضحت تشابه مع الفطر كوليتوتريكم كلورفيتى بنسبة ٩٩ و١٠٠% عند مضاعفة ITS1 و ITS4 . وبالإضافة لذلك تم تقييم بعض وسائل المقاومة الكيماوية حيث تمكنت معاملة الرى بمحلول المبيد soil drench (مرة واحدة عند الزراعة) من تثبيط الإصابة بنسبة وصلت إلى ٧٣.٩% باستخدام مبيد يونيفورم ٣٩% بمعدل ٦٥٠ سم^{3}/فدان. حيث خلصت الدراسة إلى أن أنواع جنس الكوليتوتريكم غير متخصصة تماماً. وأن استخدام طرق التعريف المتطورة (PCR) جنبا إلى جنب مع الصفات المورفولوجية أصبح ضروريا للوصول إلى تعريف دقيق. كما أن تطبيق المعاملة الكيماوية بالرى بمحاليل المبيدات يمكن أن يكون وسيلة لخفض نسب حدوث إصابة القرعيات بمرض الإنثراكنوز. وتعد هذه الدلالات بالإضافة إلى نتائج المدى العوائلى نتيجة هامة يمكن وضعها فى الاعتبار عند وضع برامج التربية فى الكنتالوب لمقاومة مرض الأنثراكنوز.