Detection of *Pseudomonas syringae* pv. *tomato* coronatine toxin by polymerase chain reaction (PCR)

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ABSTRACT

Sixteen bacterial isolates of *Pseudomonas syringae* pv. *tomato* (Okabe) Younis *et. al.*, the causal agent of bacterial speck of tomato, were isolated and identified from diseased tomato samples collected from certain tomato growing areas in West Delta. The 650 bp coronatine genes cluster was detected in all tested *Ps. syringae* pv. *tomato* isolates by using two 17-bp oligonucleotide primers; primer 1 and primer 2 by PCR analysis. For the detection of the variations among the tested isolates of *Ps. syringae* pv. *tomato*, the 650 bp PCR products of the six different isolates which represent the six different locations, were digested with five restriction enzymes namely; *PstI, ClaI, SmaI, CfoI* and *HaeIII*. Fragments of identical sizes were obtained from these isolates, which pro that this gene cluster is highly conserved. Moreover, *Ps. syringae* pv. *tomato* was found to be translocated systemically throughout the infected tomato shoots, since this pathogen was detected by direct isolation on KB medium followed by pathogenicity tests and by PCR analysis from the sap of symptomless tomato plant shoots. Furthermore, for the detection of systemic infection, the results indicated that the detection of bacterial speck of tomato, using PCR amplification of coronatine gene is highly efficient and more rapid than direct isolation on KB medium followed by pathogenicity tests. The pathogen was detected by PCR, 14 days after inoculation, while it was isolated 21 days after inoculation when use the direct isolation on KB medium was used followed by pathogenecity tests. The main objective of our study was detected on coronatine which is considered one of the virulence factors of the Ps. *syringae pv. tomato*

Key words: Pseudomonas syringae pv. *tomato*, coronatine, systemic infection.

Introduction

Different bacterial diseases ha been reported to attack tomato in Egypt; namely tomato speck caused by *Pseudomonas syringae* pv. *tomato* (*Ps. syringae* pv. *tomato*) (Okabe) (Young *et al.* 1992), bacterial leaf spot caused by *Ps. syringae* pv. *syringae.*, Van Hall, (Khlaif, 1991), bacterial wilt caused by *Clavibacter michiganensis* subsp. *michiganensis* and bacterial stem rot caused by *Erwinia caratovora* pv. *caotovora* Jones (Khlaif, 1993). Bacterial speck of tomato caused by *Ps. syringae* pv. *tomato* attacks tomato leas, fruits and stems, making production of disease free seedlings ry difficult and drastically affects the yield (Goode and Sasser, 1980). Within the last decade bacterial speck of tomato has become a serious problem in many tomato production areas around the world. Early infection

may reduce the yield and delay the maturity of fruits. However, the most destructive aspect of the disease is the reduction of fruit quality due to lesions formed on the fruit surface making them unmarketable especially the processing tomatoes (Getz *et al.*, 1983). The disease was reported to reduce the fruit quality by 75% in the early infection, while in the late infection, the reduction was 5% (Yunis *et al.*, 1980).

The tomato pathogen *Pseudomonas syringae* pv. *tomato*, along with several other *P. syringae* pathovars, produce coronatine, a phytotoxin that causes leaf chlorosis and plant stunting. Infection of the host plants by these bacteria induces chlorosis on the leas due to the production of coronatine. In addition, coronatine distorts leaf growth, inhibits root elongation, and causes hypertrophy when applied to the cut surface of potato tubers. The mechanism of action of coronatine is complex, and is due in part to the resemblance between coronatine and the plant signaling molecule methyl jasmonate (Mitchell, *et al.*, 1983 , Nishiyama, and Ezuka, 1978 and Wiebe,1993).

Greenhouse studies with Tn*5*-induced Cor2 mutants of *P. syringae* pv. *tomato* ha shown that bacteria possessing this virulence factor achieve higher population levels and produce larger lesions on their host plants (Bender, *et al.,*1987 and Moore, *et al.,* 1989) . Toxin production thus appears to give this pathogen a selective advantage in its natural habitat. Recent studies ha demonstrated that coronatine production is controlled by a gene cluster that is at least 30 kb in size (Bender, *et al.*, 1993, Ma, *et al.*, 1991 and Young, *et al.*; 1992) . To date, coronatine is the only bacterial phytotoxin that has been shown to be under the control of plasmid-borne genes (Coplin,. 1989., Kinscherf, *et al.*,1991 and Quigley, *et al.*,1985). However, plasmid control of coronatine synthesis is not universal; there is one known *P. syringae* pv. tomato strain, DC3000, which carries the *cor* gene cluster on the chromosome (Moore, *et al.*, 1989).

Materials and Methods

Locations of the collected samples

Diseased tomato samples infected with bacterial speck were collected from certain locations including: Sabahia research station, Faculty of Agriculture Research farm of Faculty of Agric. at Abies, El Banger, village 4 , 13, El Bostan and Kafer El- dwar, West Delta Egypt.

Isolation and identification of the causal agent

Raised, circular, smooth, glistrn fluorescent colonies were developed on KB plates when the surface of these plates was inoculated with the suspension of the macerated tomato plant samples.

Detection of Coronatine gene cluster from *Ps. syringae* pv. *tomato* culture:

Genomic DNA extraction from bacterial culture

One ml of or night culture of each of the different obtained pathogenic *Ps. syringae* pv. *tomato* isolates was placed in 1.5 ml eppendorff tubes. The tubes were centrifuged at 13000 rpm for 2 mins to pellet the cells and discarded the supernatant. The pellets of each isolate were resuspended in 600 µl of nuclei lysis solution, the tubes were incubated at 80°C for 5 mins to lyse the cells then cooled to room temperature. A total of 3 µl of RNase solution were added to the cell lysate and the tubes were inverted 2-5 times to be mixed, and incubated at 37°C for 30 mins. After cooling the samples were transferred to room temperature, 200 µl of protein precipitation solution were added to the RNase-treated cells lysate and vortexed vigoursly at high speed for 20 secs to mix the protein precipitation solution with the cell lysate. Tubes were incubated in ice for 5 mins then centrifuged at 13000 rpm for 3 mins. Supernatants containing the DNA were transferred to a clean 1.5 microcentrifuge tube containing 600 µl of (99%) isopropanol, and gently mixed by inversion until the thread like strands of DNA form a visible mass. Tubes were centrifuged at 13000 rpm for 2 mins. Carefully the supernatants were discarded and the tubes were drained on clean absorbent papers. Pellets were resuspended in 600 µl of (70%) ethanol at room temperature and gently inverted the tubes several times to wash the DNA pellets, followed by centrifugation at 13000 rpm for 2 mins, and carefully the ethanol was aspirated. The tubes were drained on a clean absorbent paper and allow the pellets to air dry for 10-15 mins. Finally 100 µl of DNA Dehydration solution were added to each tube and stored at 4°C (Genomic DNA purification kit, Promega).

PCR amplification

Two oligonucleotide primers with the sequence; Primer 1 (5'-GGCGCTCCCTCGCACTT-3') and primer 2 (5'-GGTATTGGCGGGGGTGC- 3') were used for the coronatine gene cluster amplification.

The PCR was carried out in a total volume of 25 µl, containing: 25 µl (10X) Taq DNA polymerase buffer containing (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 1.5mM $MgCl_2$, 0.1% Triton X-100,), 0.2 mM (µl) of the four dNTPs mixture, 25pM (1 µl) of each primer, 0.5 U (0.1 µl of 5 U/ µl) of Taq DNA polymerase, and 2 µl of template DNA.

PCR protocol

Amplification reaction was performed in a thermal cycler (9700 Perkin- Elmer) in which amplification includes 37 cycles using the following protocol: denaturation at 93°C (in the first cycle for 2 mins. and in subsequent cycle for 1 min.), annealing of the primers to the template DNA at 63°C for 2 mins. and polymerization at 72°C for 2 mins. and finally an additional extention at 72°C for 10 mins. After amplification the expected PCR products were stored at 4°C until electrophoresis.

Gel electrophoresis:

Ten microliters of the PCR product mixed with 2 µl of bromophenol blue as loading dye (6X), were separated by horizontal agarose electrophoresis in 1X TBE (Tris Borate EDTA) buffer (Promega), 1.5% (W/V) LE agarose (Promega) containing 1 µl of ethidium bromide per 10ml of running buffer. The 100bp ladder marker (Promega) was used. Electrophoresis reaction was performed for 120 mins at 100V, and then gels were photographed under UV light (302nm).

RFLP analysis of PCR product

Three microliters of the PCR product were digested for four hours with the following restriction endonucleases: *PstI, ClaI, SmaI, CfoI, and HaeIII* (Promega). The reactions were performed in a final volume of 20 µl, with 5 U (0.5 µl of 10 U/ µl) of each enzyme, 1X (2 µl of 10 X) of each specific enzyme buffer, and 2 µg (0.2 µl of 10 µg/ µl) of acetylated BSA . The assay temperature for all mentioned restriction enzymes was 37°C, except SmaI was 25°C.

Digested products were separated by horizontal electrophoresis in 1X TBE buffer using a 2% (w/v) LE agarose (Promega) containing 1 µl of ethidium bromide per 10ml of running buffer. In each well, 10 µl of the digested products were mixed with 2 µl of bromophenol blue as loading dye (6X), also uncut of PCR product was loaded as a control. The 100 bp ladder (Promega) was used as a molecular marker. Gels were run against 75V for 120 mins, and photographed under UV light (302 nm).

Detection of the systemic infection in the infected tomato seedlings

DNA extraction from tissues of tomato plants

DNA was extracted from plant tissues of infected tomato seedlings with reference culture at different periods post inoculation; 10 days, two, three and four weeks, to detect the possible systemic infection or latent infection of *Ps. syringae* pv. *tomato*. Four samples of 5cm, 2cm below the tips, were collected at the different mentioned ages, each sample consists

of five symptomless tomato shoots. All tomato shoots before maceration were surface disinfected with 2.5 % chlorax solution for 1 min, washed with sterile distilled water to be free of surface contaminations. Then the following procedure was followed:

Firstly, direct isolation from the obtained extracts of the macerated tissues on KB medium, followed by identification of the obtained culture as mentioned previously.

Secondly, using one ml from the macerated tissues extracts for DNA isolation as described by Edwards *et al.*, (1991), and modified by Marti *et al.*, (1998), Cubero *et al.,* (1990),and Liop *et al.,* (1999), in which the extract was placed in a 1.5 ml eppendorff tubes, centrifuged at 13000 rpm for 5 mins, then the supernatants were discarded and the pellets were resuspended in 500 µl filter sterilized extraction buffer containing : 200 mM Tris-HCl, 250mM NaCl, 25 mM EDTA, 0.5% SDS , 2 % PVP-15, pH 7.5. The tubes were shaken at room temperature for 1 hr, and then heated at 95°C for 5 mins for cell lysis then centrifuged at 5000 rpm for 5 mins, 450 µl of the supernatants of each extract were taken into new eppendorff tube containing 450 µl of 99% isopropanol. Tubes were mixed gently and left for one hour at room temperature. The mixture was centrifuged again at 13000 rpm for 10 mins, and the supernatants were removed, then the pellets were air dried at room temperature for one hour by inverting the tubes on a sterile filter paper. Finally the pellets were resuspended in 100 µl of nuclease free water and stored at – 20 °C to be used for PCR assays.

Detection of Coronatine gene cluster from plant by PCR

Five microliters of DNA extracts were used as a template for coronatine amplification as mentioned above. The product was detected by electrophoresis as described earlier. DNA was extracted from tissues of tomato plants artificially inoculated with reference culture, and from healthy tomato plants to ser as check.

The detection of the systemic infection by PCR assays was compared with direct isolation on king B medium, to evaluate the sensitivity of PCR technique.

RESULTS

Data in Table 1 show that the different 16 isolates reacted negatively to: oxidase, potato soft rot, and the arginine dihydrolase tests. Dome shaped colonies were produced from each isolate on NA medium supplemented with 5 % sucrose. Moreover, the isolates produced hypersensitive reaction in the inoculated tobacco leas.

The reactions of the isolates to the different biochemical tests were identical to that of the reference culture of *Ps. syringe* pv. *tomato* DeN3.

Pathogenicity test

Table 1 showed that the tested isolates were found to be pathogenic and small specks surrounded by yellow halo were developed on the leas of GS 12 tomato cultivar, when tomato leas were sprayed with 10^8 CFU/ml of *Ps. syringae* pv. *tomato* symptoms were similar to those developed on tomato leas inoculated with the suspension of the reference culture of *Ps. syringae* pv. *tomato* DeN3 and signs of infection more detected on tomato leaves sprayed with sterile water.

Detection of the coronatine gene cluster from bacterial cultures

The coronatine gene cluster was detected in the DNA extracts of the 16 tested isolates of *Ps. syringae* pv. *tomato* grown on KB medium, and formed the expected PCR product of 650 bp (Fig. 1).

PCR-RFLP analysis of coronatine gene cluster

Digestion of the amplified PCR product of the coronatine gene cluster for six isolates obtained from the six different locations, using fi different restriction enzymes, resulted in identical bands formation for each enzyme. The results showed that the enzymes digested the amplified product into restriction fragments of expected sizes; 400 bp and 220 bp for *PstI* (2), 430bp and 230 bp for *ClaI* (Fig.3), 350 bp and 310 bp for *SmaI* (Fig. 4), 320 bp, 200 bp and 100 bp for *CfoI* (Fig. 5), and 290 bp, 180 bp and 120 bp for *HaeIII* (Fig. 6).

Detection of the Coronatine gene cluster from symptomless plant tissues

Results of the coronatine gene amplification using DNA extracts from apparent healthy shoots taken from seedlings artificially inoculated with reference culture *Ps. syringae* pv. *tomato* DeN3, were compared with direct isolation on KB medium and pathogenicity test. The pathogen was isolated directly on KB medium 21 days after inoculation with weak growth, and 28 days after inoculation with dense growth. The obtained cultures were identified by biochemical tests and pathogenicity test. While by PCR the 650 bp of the coronatine genes cluster produced by *Ps. syringae* pv. *tomato* was detected 14 days after inoculation with a faint band, , and with clear bands after 21 and 28 days of inoculation from the plant sap (Fig.7).

Table (1): Locations, biochemical tests, pathogenicity test, and PCR coronatine gene cluster detection of *P. syringae pv tomato* isolates collected from certain tomato growin areas.

Isolate no	location	Biochemical tests (LOPAT)					Pathogenicity	Detection of coronatine
		Levan	Oxidase	Potato rot	Arginine dihydrolase	Hypersensiti reaction		
1-3	Sabahia research station	+	-	-	-	+	+	+
4-6	Farm of Faculty of Agriculture	+	-	-	-	+	+	+
7-8	El Banger, village 4	+	-	-	-	+	+	+
9-10	El Banger, village 13	+	-	-	-	+	+	+
11-13	El Bostan	+	-	-	-	+	+	+
14-16	Kafer El- dwar	+	-	-	-	+	+	+
Reference culture (T1D6)		+	-	-	-	+	+	+

+ = Positive   - = negative

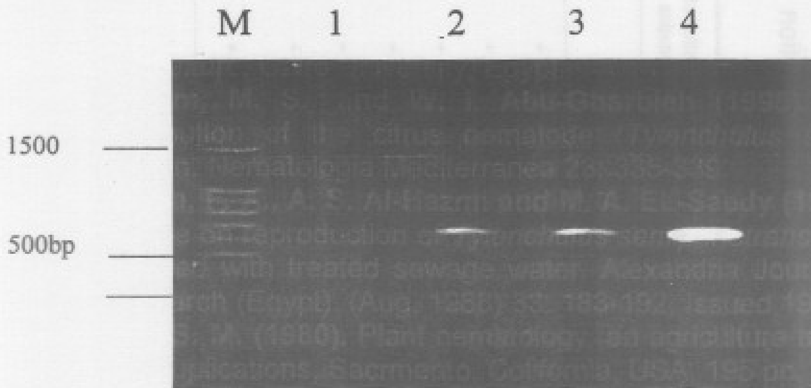

Fig. (1): PCR detection of coronatine gene cluster from bacterial DNA: Lane M, 100 bp molecular size marker, Lane 1, Water control, Lane 2, Positive control using DNA extracted from bacterial culture of reference Ps. syringae pv. tomato, Lane 3, Positive result using 2 µl of DNA extracted from isolated bacterial culture, Lane 4, Positive result using 5 µl of DNA extracted from isolated bacterial culture.

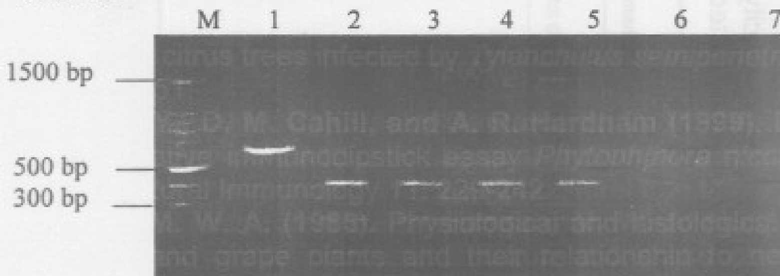

Fig. (2): Restriction pattern of coronatine gene cluster after digestion with *PstI* enzyme *Lane M*, 100 bp molecular size marker, *Lane 1*, Uncut PCR product (Sabahia), *Lane 2*, Sabahia isolate, *Lane 3*, F.Ag. Farm isolate, *Lane 4*, village 4 isolate, *Lane 5* village 13 isolate, *Lane 6*, El Bostan isolate, *Lane 7*, Kafer El- dwar isolate.

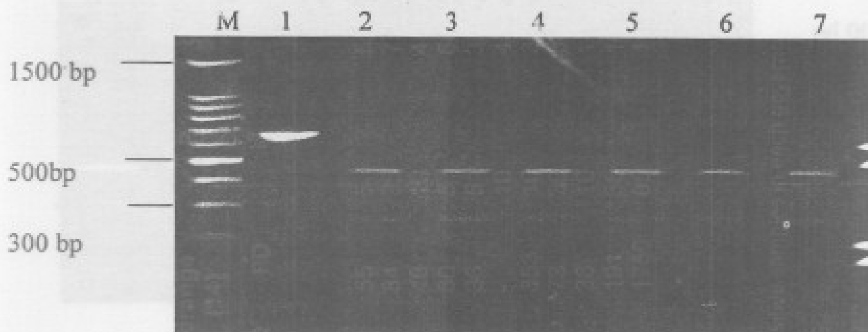

Fig. (3): Restriction pattern of coronatine gene cluster after digestion with *ClaI* enzyme *Lane M*, 100 bp molecular size marker, *Lane 1*, Uncut PCR product (F.Ag. Farm), *Lane 2*, Sabahia isolate, *Lane 3*, F.Ag. Farm isolate, *Lane 4*, village 4 isolate, *Lane 5* village 13 isolate, *Lane 6*, El Bostan isolate, *Lane 7*, Kafer El- dwar isolate.

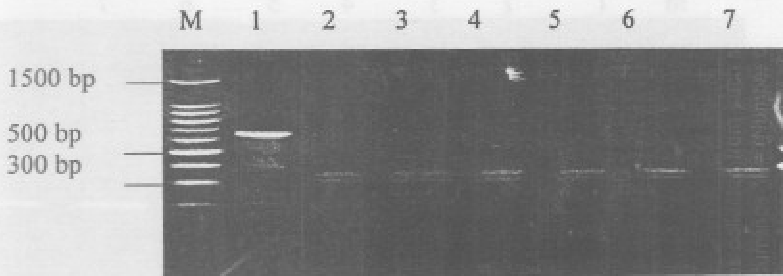

Fig. (4): Restriction pattern of coronatine gene cluster after digestion with *SmaI* enzyme *Lane M*, 100 bp molecular size marker, *Lane 1*, Uncut PCR product (village 4), *Lane 2*, Sabahia isolate, *Lane 3*, F.Ag. Farm isolate, *Lane 4*, village 4 isolate, *Lane 5* village 13 isolate, *Lane 6*, El Bostan isolate, *Lane 7*, Kafer El- dwar isolate.

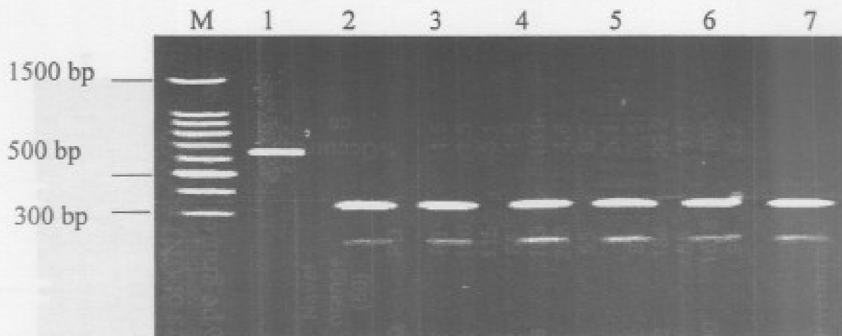

Fig. (5): Restriction pattern of coronatine gene cluster after digestion with *CfoI* enzyme *Lane M*, 100 bp molecular size marker, *Lane 1*, Uncut PCR product (village 13), *Lane 2*, Sabahia isolate, *Lane 3*, F.Ag. Farm isolate, *Lane 4*, village 4 isolate, *Lane 5* village 13 isolate, *Lane 6*, El Bostan isolate, *Lane 7*, Kafer El- dwar isolate.

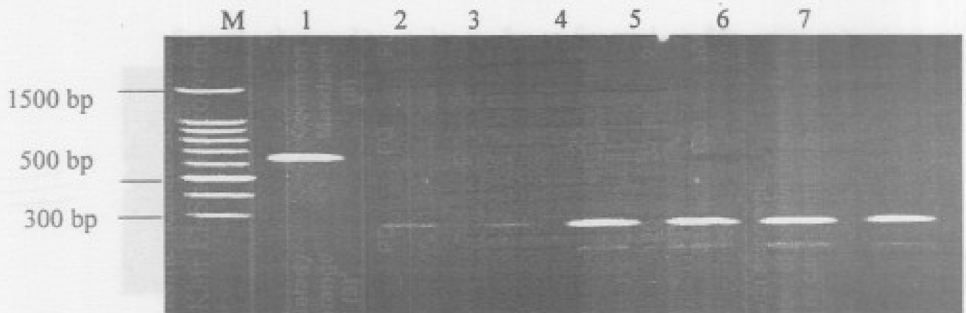

Fig.(6): Restriction pattern of coronatine gene cluster after digestion with *HaeIII* enzyme *Lane M*, 100 bp molecular size marker, *Lane 1*, Uncut PCR product (El Bostan), *Lane 2*, Sabahia isolate, *Lane 3*, F.Ag. Farm isolate, *Lane 4*, village 4 isolate, *Lane 5* village 13 isolate, *l ane 6*, El Bostan isolate, *Lane 7*, Kafer El- dwar isolate.

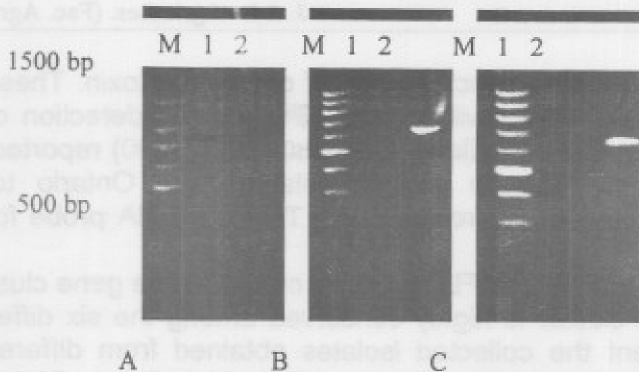

Fig. (7): PCR detection of coronatine genes cluster from tomato plant DNA,14 days (A), 21 days (B), 28 days (C), after inoculation:*Lane M*, 100 bp molecular size marker, *Lane 1*, 3, 5, showed negative control using DNA from healthy tomato plant, *Lane 2, 4, 6,* showed band (650 bp) of coronatine genes cluster from plant DNA extracted after 14, 21, 28 days of inoculation.

DISCUSSION

The reaction of different bacterial isolates, collected from the certain tomato growing areas including nurseries and open fields, to the biochemical tests carried out in this study were similar and identical to the reactions of the reference culture of *Ps. syringae* pv. *tomato* (DeN3). This confirms that the causal agent of bacterial speck of tomato in the inspected fields of West Delta, Egypt is *Ps. syringae* pv. *tomato* (Okabe), as reported by Sara,*et al.*, (2005). The results of this study indicated that the disease occur in certain tomato growing areas tested in West Delta, including Sabahia research station. Farm of Faculty of Agriculture, El Banger, village 4 , 13, El Bostan and Kafer El- dwar., since pathogenic *Ps. syringae* pv. *tomato* isolates were isolated from diseased tomato samples collected from the above mentioned locations.

The presence or absence of the bacterial speck of tomato in the surveyed locations of west delta could be explained by that the disease was transmitted by infected seedlings.

A PCR assay was used in this study for the detection of *Ps. syringae* pv. *tomato* in infected tomato plants. The PCR was followed the direct isolation on KB medium and pathogenicity tests for the isolates obtained from naturally infected tomato plants. The detection of coronatine genes cluster by PCR for the collected isolates of *Ps. syringae* pv. *tomato,* was found to be useful to determine the potential pathogenicity, because all isolates that induce speck symptoms on artificially inoculated plants also

produced the 650 bp expected band of coronatine toxin. These results are in agreement with Bereswill *et al.* (1994), for the detection of coronatine gene cluster by PCR. Similarly, Cuppels *et al.*, (1990) reported that all 244 *Ps. syringae* pv. *tomato* strains isolated from Ontario tomato fields produced coronatine and reacted with TPR1, a DNA probe for coronatine producers.

The results of PCR-RFLP analysis of coronatine gene cluster indicated that this gene cluster is highly conserved among the six different isolates which represent the collected isolates obtained from different locations, using five different endonucleases (*PstI, ClaI, SmaI, CfoI, and HaeIII*), where identical bands were obtained for the different isolates using the different enzymes. These results confirms the results obtained by Bereswill *et al.* (1994) who reported that the coronatine gene cluster gave identical fragments when they were digested with *PstI, ClaI, and SmaI*, and they concluded that the sequence analysis of coronatine gene cluster prod to be valuable in showing relatedness between strains and pathovars of *Ps. syringae*, also they reported that en geographical isolation of *Ps. syrinae*

pv. *glycinea* that produce coronatine gave identical sequences of this gene cluster. In this study, also the use of the two other restriction enzymes (*CfoI and HaeIII*), confirm the conservative type of this gene cluster.

Additional work such as the use of other restriction enzymes and the sequencing of the coronatine gene cluster of each isolate will ascertain that this gene cluster is highly conserved in Egyptian isolates of *Ps. syringae* pv. *tomato*.

Although our results showed that the coronatine which is considered one of the virulence factors of the *Ps. syringae* pv. *tomato*, is highly conserved, the variation in the severity of the bacterial speck disease of tomato was observed, could be related to the presence of other virulence factors of this pathogen such as virulence proteins secreted via type III secreted system (Charkowski, *et. al.*, 1998, Galan and Collmer, 1999, and Cornelis and van Gijsegem, 2000).

The coronatine gene cluster was detected by PCR amplification directly from DNA extracts of symptomless tomato shoots. Results of this study showed that the *Ps. syringae* pv. *tomato* translocated systemically throughout the tomato plants, and this indicates that the *Ps. syringae* pv. *tomato* is a seed-borne pathogen as reported by McCarter *et al.*, (1983), also these results are in agreement with Kim (1979) who reported that the speck disease was found in fields planted with symptomless tomato plants. In our study the detection of the systemic infection of the pathogen in symptomless tomato shoots through the detection of coronatine gene

cluster by PCR, ha a great impact on the farmers for production of disease free seedlings, because symptomless tomato seedlings infected with this disease could develop epidemic situation in fields under favorable environmental conditions.

Moreover, the detection of the coronatine gene cluster by PCR amplification directly from DNA extracts of symptomless plant shoots was compared with direct isolation on KB medium and pathogenicity test. The results showed that the detection of coronatine gene cluster by PCR amplification was more efficient and highly sensitive than the use of conational methods; direct isolation on KB medium and pathogenicity symptoms, since the pathogen was isolated from plant sap after 21 days of inoculation while it was detected from the plant sap after 14 days of inoculation through the detection of coronatine gene cluster by PCR. These could be related to that general and selective media are not highly efficient in the detection of low population of the pathogen while PCR could require low population to detect the coronatine gene cluster or the latent infection.

Detection of *Ps. syringae* pv. *tomato* by PCR is important because the bacterium is resistant to copper compounds. Different copper compounds were previously used to suppress the disease incidence and severity, such as Cupric hydroxide, Kosid 101, and Cupral, but none of the tested compounds found to be highly effective in controlling the bacterial speck disease of tomato (Conlin and McCarter, 1983, Abo-Elsamen, 1993). Also Ullrich, et. al., (1993) found that there is a connection between copper resistance and synthesis of coronatine in *Ps. syringae* pv. *glycinea* that causes bacterial blight of soyabean.

Since all isolates in this study produce the phytotoxin coronatine, therefore the detection of *Ps. syringae* pv. *tomato* by PCR is rapid and effective method at transplanting industry, allowing the farmers to be alert for taking proper precautions for minimizing the effects of this disease, also disease free seedlings must be distributed to prevent spreading of the disease to different tomato growing areas.

Detection of systemic infection of *Ps. syringae* pv. *tomato* could bring a new dimension in the epidemiology of the disease since apparent healthy transplants could be distributed to the farmers and under favorable environmental conditions, the disease could result in destruction of the crop and reducing the yield.

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الملخـــــص

الكشف عن سم الكروناتين للبكتيرة سدوموناس سيرنجي في الطماطم وذلك باستخدام تفاعل البلمرة المتسلسل

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تم عزل ستة عشر عزلة بكتيرية لمسبب مرض التبقع البكتيري على الطماطم, وذلك من عينـات كانت تظهر عليها اعراض المرض جمعت من مناطق مختلفة لزراعة الطماطم فى غرب الدلتا. وقد تـم تعريف هذه العزلات بيوكيميائيا, وبواسطة اختبار قدرتها الامراضية على الطماطم على انهـا البكتيريـا *Pseudomonas syringae pv. tomato*

كما تم الكشف عن السم النباتي coronatine في جميع هذه العزلات. حيث استخدمت طريقـة تفاعلات البلمرة المتسلسلة لتكثير مجموعة الجين المسؤولة عن انتاج هذا السم, وقد ثبت وجود مجموعـة الجين في جميع العزلات المذكورة.

وللكشف عن وجود سلالات مختلفة لهذا المرض واعتمادا على مجموعة الجين المـسؤولة عـن انتاج السم, فقد تم استخدام خمسة انزيمات مقطعة وهي,*HaeIII* (*PstI, ClaI, SmaI, CfoI*), حيـث لستخدم ناتج طريقة تفاعلات البلمرة المتسلسة لست عزلات مختلفة مثلت ست مواقع مختلفة جمعت منهـا العزلات المرضية. وقد اعطت الانزيمات نتائج متشابهة لكل انزيم على للعزلات الست المختبرة, وهـذا يبين ان مجموعة الجين لهذه العزلات محفوظة.

وجد ان المسبب المرضي لمرض التبقع البكتيري على الطماطم ينتقل جهازيـا خـلال عـصارة النبات. حيث تم الكشف عن المسبب المرضي في سيقان نباتات الطماطم المعدية اصطناعيا, والتي لا تبدي اعراض ظاهرية للاصابة, وذلك بالعزل على بيئة غذائيةKB وباجراء العدوى الاصطناعية على نباتـات طماطم, لستخدام طريقة تفاعلات البلمرة المتسلسلة. وقد اشارت النتائج على ان التاكـد مـن الاصـابة الجهازية بأستخدام طريقة تفاعلات البلمرة المتسلسلة كان اكثر كفاءة واسرع في الكـشف عـن المـسبب المرضي من الطرق التقليدية. حيث تم الكشف عنه بواسطة طريقة تفاعلات البلمرة المتسلسلة بعد ١٤ يوم من الاصابة بينما تم عزله واثبات قدرته الامراضية بعد ٢١ يوم بعد اجراء العدوى الصناعية.