

## EVALUATION OF THE POLYMERASE CHAIN REACTION (BIO-PCR) , ELISA AND SEMISELECTIVE MEDIUM FOR DETECTION TOMATO BACTERIAL CANKER.

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Current seed health testing is based on standardized methods described by International Seed Testing Association (ISTA). Some of these tests are time consuming. Most techniques being used for detecting seedborne bacteria utilize semiselective agar media for detecting the pathogen followed by pathogenicity tests for identification. These techniques can be very sensitive but depend upon the selectivity of the medium and contamination level of the samples with saprophytes. Semiselective agar media have a sensitivity range between  $1 \times 10^1$  to  $1 \times 10^2$  cfu/ml. This is 100-1,000 fold more sensitive than ELISA. Serological techniques (ELISA) have the advantage of producing quicker results but have the disadvantage of a relatively low detection threshold around  $1 \times 10^5$  cfu/ml. Semiselective agar media have several disadvantage, including overgrowth of the target bacterium by saprophytic bacteria contaminated the seed, antibiotics produced by saprophytes, and time consuming. Molecular diagnostics offers the potential for sensitive and specific detection of *Clavibacter michiganensis subsp. michiganensis* (CMM). Advantages of BIO-PCR, over the existing PCR techniques include the elimination of false positives results due to the presence of dead cells that may be present on the tomato seeds, elimination of false negatives results due to potential PCR inhibitors in seed extracts and increased sensitivity of detection.. The BIO- PCR technique is specific for CMM without amplification the closely related bacterium *Clavibacter michiganensis subsp. sepedonicus*, or other bacterial species. The assay was successfully applied to the detection of CMM in tomato seeds and was sensitive to approximately 10 cells per PCR assay. The described PCR method for identification of this pathogen is very fast (1-2 days) and economical, due to the very small volume (10  $\mu$ l.) of the PCR reaction mixture. this method is based on amplification of the bacterial plasmid DNA fragment and may be very useful in routine identification of CMM and could be an alternative tool for diagnoses, especially for quarantine work..

**Keywords** : Tomato , bacterial canker , *Clavibacter michiganensis sub sp. michiganensis* , semiselective medium , ELISA , Bio-PCR.

## INTRODUCTION

Recently detection and rapid identification of seed-borne pathogens is the front line of defence in the control of seed-transmitted diseases due to the increased international movement of germplasm and commercial seed , which increases the chances for the inadvertent introduction of foreign plant pathogens into new regions (Schaad *et al.*,1994).

*Clavibacter michiganensis subsp. michiganensis* is a serious seedborne bacterial pathogen of tomato. It is a causative agent of bacterial canker (Chang *et al.*,1989), spread by contaminated seeds (Tsiantos,1987) and plantlets (Gitaitis,1990, Gitaitis *et al.*, 1991). Because of the rapid spread of the pathogen under suitable weather conditions, even low levels of primary infection can result in severe epidemics. Losses in the yield of infected plants (Chang *et al.*,1991) are the main reason for considering *Clavibacter michiganensis subsp. michiganensis* among the quarantine pests (EU,1992).

*C. michiganensis subsp. michiganensis* may be identified using semiselective media (Shirakawa and Sasaki,1988), also by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assays (Franken *et al.*,1993; Riley,1987). Serological methods are sensitive (Rat, 1984) but there are difficulties in obtaining sufficiently specific antisera. Specific and sensitive ELISA methods have been developed and it is claimed that they are useful in the routine analysis of latent infection (Gitaitis *et al.*, 1991; Kramer and Griesbach, 1995). Recently, PCR-based molecular probes specific for *C. michiganensis subsp. michiganensis* and its virulent strains have been

reported (Dreier *et al.*, 1995). The biological tests on tomato plants after isolation of bacteria on semiselective media are time consuming and unsuitable for large-scale application. In the recent years more and more often the polymerase chain reaction is applied for diagnosis of various bacteria (Hadidi *et al.*, 1995). The various saprophytic bacteria, which give colonies identical with those given by *C. michiganensis* subsp. *michiganensis*, are very often isolated from tomato seeds.

Based on the fact mentioned above and also on the fact of easiness and fastness of spread of *C. michiganensis* subsp. *michiganensis*, there is an urgent need to introduce sensitive and fast methods for detection and identification of this pathogen. PCR- based technique has several disadvantages when used for diagnosis, including their inability to differentiate between dead and living cells which is important in many phytosanitary applications also PCR can be inhibited by chemical compounds present in plant samples. To avoid these problems PCR can be applied in combination with isolation on media (Schaad, *et al.*, 1995).

The aim of the present work is to compare and describe the fast and the simple method for specific identification of *C. michiganensis* subsp. *michiganensis* using polymerase chain reaction (BIO-PCR).

## MATERIALS AND METHODS

### Seed stomaching liquid plating test:

Seed stomaching: Place 24 g of the seed sample (approximately 10,000 seeds) in a 20 cm x 25 cm and 0.15 mm thick plastic bag containing 100 ml sterile phosphate-Tween buffer (7.75 g/L of Na<sub>2</sub>HPO<sub>4</sub> + KH<sub>2</sub>PO<sub>4</sub> + 0.2 ml/L Tween 20), pH 7.4. Incubate the plastic bag with its contents in a refrigerator at 4°C for 15 min. After the refrigeration time, place the plastic bag with its contents in a stomacher and blend for 15 min.

Liquid plating: Pipette 0.1 ml of 0, 1:10, 1:100 dilutions (prepared using phosphate buffer without Tween) of each sample onto each three plates of modified SCM medium (mSCM Waters and Bolkan). Spread with an L shaped glass rod, and incubate at 26 °C. mSCM is prepared as follows: dissolve in 980 ml distilled water in a 2 L flask 2.62g K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 0.25 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.5 g boric acid, 10 g mannose, and 0.1 g yeast extract. Add 1 drop (1 ml pipette) of pourite and 12 g of agar, and autoclave at 121.6 °C at 0.95 Kg/cm<sup>2</sup> for 15 min. Following cooling to 45-50 °C in a water bath add 100 mg nicotinic acid (dissolved in 20 ml sterile distilled water), 30 mg nalidixic acid (sodium salt, dissolved in 1 ml of 0.1 M NaOH), and 200 mg cycloheximide (dissolved in 1ml absolute methanol) (Fatmi, and Schaad 1988). Distribute the medium into petri plates (20 ml/plate) and store plates at 4°C until needed. Examine plates after 7 and 10 days. Remove suspected colonies with a sterile transfer loop and streak onto YDC agar (10 g yeast extract, 20 g light powder CaCO<sub>3</sub>, and 15 g agar in 1 L distilled water). Incubate at room temperature (24 ± 1°C). Compare with Known culture of *Clavibacter michiganensis* subsp. *michiganensis* (CMM) on YDC.

### Identification of suspected colonies of CMM using ELISA:

Suspected CMM colonies are grown on YDC for 24-48 hr. and identity of the

colonies determined using *CMM* monoclonal antibodies and the ELISA procedure with an Agdia reagent set containing peroxidase labeled conjugates (Agdia, Inc., 30380 County Road 6, Elkhart, IN 46514, USA). Dilute the *CMM* monoclonal antibodies in coating buffer (1:1000 dilution) and load plates by adding 0.2 ml/well. Place plates in closed humid box and incubate at room temperature for 4 hr or at 4°C overnight. Remove the coating solution and wash plates by flooding wells with PBS-Tween. Repeat washing 3 times; wait 3 min. for each wash. Dilute suspected *CMM* colonies previously grown on YDC in extraction buffer and add to duplicate wells 0.2 ml/well. Use extraction buffer and a known *CMM* culture as controls Incubate plates at room temperature (24°C ± 1°C) for at least 2 hr or at 4°C overnight in Dilute *CMM* monoclonal antibodies in the extraction buffer (1:500 dilution) and load plates by adding 0.2 ml/well. Incubate plates at room temperature for 2hr in a closed humid box. Wash plates as previously described. Dilute (1mg/ml) o-phenylenediamine in substrate solution and load plates by adding 0.2 ml/well. Incubate plates at room temperature in the dark for 15-30 min. or until the positive controls develop a dark yellow-orang color.

Stop the reaction by adding 75µl. of 3M sulfuric acid. Measure optical density at 490 nm

or evaluate visually color intensity is proportional to bacterial concentration (Kramer and Griesbach, 1995).

**Biological tests:**

The biological tests were performed on the tomato plants. Young plants 2-3 true-leaf were mechanically inoculated (Carborundum was used as an abrasive) with suspected *CMM* colonies on YDC for 24-48 hr and grown in the greenhouse at 22-25°C. Symptoms are recorded after 7-11 days (Gitaitis *et al.*, 1991)..

**Bacteria and their growth:**

Five isolates of *CMM*, two isolated from naturally infected tomato seeds of Hybride Alex 63 cv. and Castel Rock cv. two isolated from seeds collected from infected plants of Hybride Alex 63 cv. and. Castel Rock cv. respectively and one Known culture of *CMM* as a control. The tested bacteria were grown on YDC-agar medium and on TBY-agar medium (Fatmi&Schaad 1988) at 27°C for 48 hour.

**Nucleic acids extraction:** The bacterial DNA was isolated from bacteria grown in the media. One colony was suspended in 100 µl of water, boiled for 10 min. at 99°C, cooled for 10 min. at 4°C and then centrifuged for 5 min. at 10000 rpm at room temperature. The supernatant was used as a template for PCR. The nucleic acids prepared in such a way were stored at - 20°C (Dreier *et al.*, 1995).

**Polymerase chain reaction:**

One pair of specific oligonucleotide primers for *CMM* (1,2) and One pair of specific oligonucleotide primers for *C michiganensis subsp. sepedonicus* (3,4) from Pharmacia Biotech (Amersham Pharmacia Biotech UK Limited, England HP79NA) were tested in this experiment to amplify the template DNA.

NO. Of primer	Nucleotide sequence 5` to 3`
1	GCGAATAAGCCCATATCAA

2	CGTCAGGAGGTCGCTAATA
3	CCCGAAGAGAGCACACTACCT
4	CTCAACTCCCACGCATAAAGA

The polymerase chain reaction was performed in the thermocycler UNO-Thermoblock with heated lid suitable for 25 tubes of 0.2 ml volume each (Biometra, Germany). The final volume of one sample for PCR was 10µl. The PCR reaction mixture was added to the 4µl of previously DNA (Hadidi *et al.*,1995).

Composition of the PCR reaction mixture (for 10 µl reaction volume):

Component	Final concentration
PCR buffer (Biometra, Germany)	10 mM Tris-Hcl, pH 8.8, 1.5 mM MgCl <sub>2</sub> 50mM KCl, 0.1% Triton X-100
Thermostable polymerase PrimeZyme	0.1 unit
DATB, dCTB, dGTB, dTTB mix (PromegaUSA)	0.2 mM
PCM primer	0.2 µM
No mineral oil was overlaid	

PCR reaction profile: initial denaturation for 5 min. at 95°C, 30 cycles of amplification; denaturation for 30 sec. At 94°C, annealing for 30 sec. At 55°C and primer extension for 1 min. at 72°C. At the end of PCR, extension of amplification products was completed for 5 min at 72°C. Heated lid temperature was 100°C.

#### **Electrophoresis of PCR products:**

The PCR products were electrophoretically analysed on 1.5% agarose gels (the electrophoresis apparatus of AgaGel Mini-Biometra, Germany). 1µl of the loading dye (10%Ficoll 400, 0.25% Bromophenol Blue, 0.25% Xylene Cyanole FF) was added to the 4 µl of PCR mixture. The electrophoresis was performed in TBE buffer (working solution; 90 mM Tris, 90mM Boric acid, 2 mM EDTA), containing the ethidium bromide as the stain. The DNA marker was applied. After electrophoresis the gels were visualized using the Biometra TI-3 (Germany) transilluminator.

### **RESULTS AND DISCUSSION**

High specificity , sensitivity, and no time and costs, are the characteristics that determine the broad use of a disease diagnostic technique. This is especially important in regard to plant bacteria (Rasmussen and Reeves,1992). Difficulties are frequently encountered in isolating one genus from another. Greater difficulties may still arise in discriminating biovars, subspecies and pathovars.

Many seed-testing methods have been investigated. Semi-selective media are routinely used to isolate *C. michiganensis subsp. michiganensis* from seed extracts or from plant tissue. All *C. michiganensis subsp. michiganensis* strains tested grew more rapidly on modified Semiselective for *C. michiganensis subsp. michiganensis* (mSCM). *C. michiganensis subsp. michiganensis* colonies on Semiselective for *C. michiganensis subsp. michiganensis* (SCM) plates after 10 days are convex, irregular, mucoid with internal black flecks. On the other hand, the mSCM plates examined after 7 and 10 days. At 7 days, *C. michiganensis subsp. michiganensis* colonies on

mSCM are light grey, 2-3 mm in diam., translucent, and easily distinguishable from other mucoid colonies by the presence of many internal flecks (specks). As incubation time increases the colonies become larger and the internal flecks become yellow whereas the non- *C. michiganensis subsp. michiganensis* colonies remain small and have no internal flecks. Suspected colonies Compared to a 7-10 day-old streak of a known culture of *C. michiganensis subsp. michiganensis* on mSCM and SCM media. The SCM and mSCM media can detect infested seed lots at a rate of one infested seed per 10,000 seed sample (Fatmi, and Schaad 1988, Schaad, 1988), mSCM, however, is less inhibitory to *CMM* than SCM. Furthermore, it does not require much experience to identify colonies of *CMM* on mSCM. Comparative tests with commercial seed lots showed that more seed lots showed positive results when tested with mSCM than with SCM. Most techniques being used for detecting seedborne bacteria utilize semiselective agar media followed by pathogenicity tests for identification. The sensitivity of these techniques depend upon the selectivity of the medium and contamination level of the samples with saprophytes. The threshold limits for isolation techniques most often range from  $1 \times 10^1$  to  $1 \times 10^2$  cfu/ml.

This is 100-1,000 fold more sensitive than serological tests such as ELISA. However, when the medium fails to adequately reduce the numbers of other organisms detection limits are normally not better than  $1 \times 10^3$  cfu/ml. Semiselective agar media have several disadvantage, including overgrowth of the target bacterium by saprophytic ones present on the seed, induction of antibiotics produced by saprophytes, and time consuming. (Randhawa and Schaad, 1984). All *Clavibacter michiganensis subsp. michiganensis* strains tested gave positive reaction by ELISA. Serological methods are sensitive (Rat, 1984) but hindered with specific antisera. On the other hand, ELISA are useful in the routine analysis of latent infection (Gitaitis *et al.*, 1991; Kramer and Griesbach, 1995). (ELISA) has the advantage of producing quicker results but with low detection threshold of around  $1 \times 10^5$  cfu/ml. The biological tests performed in greenhouse on tomato plants confirmed the diagnostic aspect of PCR, but routine application of this method is too much time consuming. Recently, PCR-based on molecular probes specific for *Clavibacter michiganensis subsp. michiganensis* and its virulent strains have been applied (Dreier *et al.*, 1995). The technique, called BIO-PCR, has many advantages over standard PCR methods for routine detection of seedborne pathogens; i.e., simple and can detect living bacterial cell only. (Schaad., *et al.*, 1995) The simple method of bacterial cell wall degradation by boiling was sufficient for obtaining intact target DNA for PCR amplification. The prepared DNA may be stored at  $-20^\circ\text{C}$  for several months. The degree of specificity of primers of pCMM was tested by DNA amplification of various *CMM* isolates, obtained from tomato seeds, and the closely related to the *CMM*, isolates such as *Clavibacter michiganensis subsp. sepedonicus*, isolated from potato (Fig1).  
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**Fig 1: Detection of *Clavibacter michiganensis subsp. michiganensis (CMM)* by PCR With pCMM specific for *CMM* in natural infected seeds and inoculated plants. Arrow indicates the 1-kb size fragment of the 1 KB**

molecular marker. Lane 1 Known culture of *CMM*, Lane 2 DNA of *Cl. m. subsp. sepedonicus* as the template, Lane 3 DNA of tomato saprophytic bacteria isolated from uninoculated tomato plants as the template, Lane 4-5 *Cl. michiganensis subsp. michiganensis* (two isolates from naturally infested tomato seeds of Hybride Alex 63 cv. and Castel Rock cv. respectively), Lane 6-7 *CMM* from seeds collected from infested plants of Hybride Alex 63 cv. and Castel Rock cv. respectively.

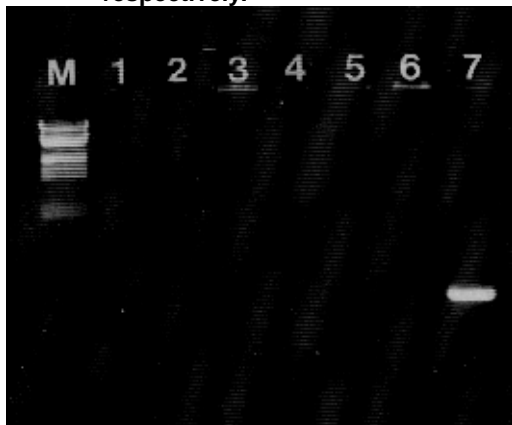


Fig 2: Detection of *Clavibacter michiganensis subsp. michiganensis* (*CMM*) by PCR with pCMS specific for *Cl. m. subsp. sepedonicus* in natural infested seeds and inoculated plants. Arrow indicates the 1-kb size fragment of the 1 kb molecular marker. Lane 1 Known culture of *CMM*, Lane 2-3 *Cl. michiganensis subsp. michiganensis* (two isolates from naturally infested tomato seeds of Hybride Alex 63 cv. and Castel Rock cv. respectively), as the template, Lane 4-5 *CMM* from seeds collected from infested plants of Hybride Alex 63 cv. and Castel Rock cv. respectively. Lane 6 DNA of tomato saprophytic bacteria isolated from uninoculated tomato plants as the template, Lane 7 DNA of *Cl. m. subsp. sepedonicus* as a template.

The expected amplification products (414 bp) were obtained only in the case when DNA extracts from *CMM*, were used. The Fig 2 shows also the specificity of primers pC. *m. subsp. sepedonicus* for detection of *C. michiganensis subsp. sepedonicus*. The expected products (201 bp) were obtained only when the preparations of DNA from *C. michiganensis subsp. sepedonicus* were used as the template for PCR. Apart of specificity, also the sensitivity of the PCR was very high. DNA from 200-300 bacterial cells for 1ml. was sufficient for obtaining the specific product of amplification. The very high sensitivity is probably due to the fact that the amplified fragment of DNA obtained from *CMM* is localized on the plasmid (Dreier *et al.*,1995). Recently, only a few experiments have been published on the usage of PCR technique for detection and identification of pathogenic plants bacteria. The PCR as the diagnostic tool was elaborated for *Agrobacterium* (Dong *et al.*,1992), *Xanthomonas campestris* (Leite *et al.*,1992; Hartung *et al.*,1992),

*Pseudomonas* sp (Gill *et al.*,1992) and *Erwinia* sp. (Bereswill *et al.*,1992; Blakemore *et al.*,1992; Smid *et al.*,1995). Also there are a few reports regarding the detection by PCR of *C. michiganensis* subsp. *sepedonicus* (Schneider *et al.*,1993; Firrao and Locci,1994) and the *C. michiganensis* subsp. *sepedonicus* (Dreier *et al.*,1995; Ghedini and Fiore,1995).

The methods based on PCR are very useful for diagnosis and identification of bacteria especially for their specificity, sensitivity and fastness (one or two days). The method of bacterial DNA amplification described in this work is very economic. The performance of one PCR reaction in the total volume of 10 µl (usually 50-200 µl) consumed low quantity of enzymes and other reagents used. According to the above mentioned, the PCR technique could be a good, alternative diagnostic tool for bacterial diagnosis, especially for quarantine work.

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## تقييم حساسية كل من اختبارات PCR الحيوى والأليزا والبيئات شبة الاختيارية فى تعريف البكتيرية *Clavibacter michiganensis* subsp. *michiganensis* المسببة لمرض التقرح البكتيرى فى الطماطم

محمد رفعت رسمى

معهد بحوث أمراض النبات - مركز البحوث الزراعية

تعتمد اختبارات صحة وسلامة البذور حالياً على طرق قياسية موحدة تحددتها الجمعية العالمية لاختبارات البذور . وتعد بعض هذه الاختبارات بطيئة وغير حساسة ومجهدة . معظم التقنيات المستعملة فى تعريف البكتيريات المصاحبة للبذور تستخدم البيئات شبة الاختيارية المتبوعة باختبارات العدوى الصناعية و يمكن أن تكون هذه التقنيات حساسة جداً ولكن تعتمد حساسيتها على انتقاء البيئة الملائمة لعزل البكتيره تحت الدراسة ومدى تلوث العينات بالبكتيريات الأخرى. وقد تراوحت حساسية البيئات شبة الاختيارية بين ١٠ الى ١٠٠ خلية فى المليليتر وهذه تعادل مائة الى ألف ضعف حساسية اختبار الأليزا ، أما الاختبارات السيرولوجية والمتمثلة هنا باختبار الأليزا فيعد من أهم مميزاتها الحصول على نتائج سريعة ولكن يعيبها الانخفاض النسبي لحساسيتها حيث بلغت ٥٠٠ خلية بكتيرية فى المليليتر إذا ما قورنت بطريقة البيئات شبة الاختيارية ، وقد ظهر عديد من العيوب لطريقة البيئات شبة الاختيارية والتي تتمثل فى النمو الزائد والمتداخل وأنتاج المضادات الحيوية لبعض البكتيريات المصاحبة للبذور والتي تعوق من عملية التعريف ونمو البكتيرية تحت الدراسة ، كما أنها تحتاج لوقت طويل لأجراء عملية التعريف وقد أظهرت التعريفات الجزيئية الحساسة الفائقة والمتخصصة للكشف عن البكتيرية

*Clavibacter michiganensis* subsp. *michiganensis* (CMM) و من أهم المميزات لطريقة PCR الحيوية مقارنة بالطرق التقليدية هو التغلب على النتائج الموجبة الكاذبة والتي ترجع لوجود الخلايا الميتة والتي تصاحب بذور الطماطم وكذلك التغلب على النتائج السالبة الناتجة عن بعض المثبطات المصاحبة لمستخلص البذور ويعد إختبار PCR متخصصاً ومحدداً لبكتيريا CMM ولا يؤدي إلى أى تكبير فى شريط الـ DNA للبكتيرية *Clavibacter michiganensis* subsp. *sepedonicus* وثيقة الصلة بالبكتيرية CMM كما لا يعمل على تكبير شريط DNA لأى من الأجناس البكتيرية الأخرى ويلعب إختبار الـ PCR حساسية فائقة لتعريف ١٠ خلايا بكتيرية كما يعد إختبار PCR سريعاً فى إجراءه إذا ما قورن بالطرق السيرولوجية (الأليزا) أو طريقة البيئات شبة الاختيارية حيث أستغرق إجراءه يوم أو يومين ، إضافة الى استخدام ١٠ ملليليتر فقط من مخلوط التفاعل . وقد أظهرت النتائج أن الطريقة المستندة على تكبير شريط DNA تكون مفيدة فى اختبارات التعريف الروتينية للبكتيره CMM من بين العديد من البكتيريات المصاحبة لبذور الطماطم وتعد طريقة سريعة للتعرف خاصة لأعمال الحجر الزراعي .