THAI AGRICULTURAL STANDARD

TAS 9031 – 2008

DIAGNOSTIC PROTOCOLS FOR *Pantoea stewartii* subsp. *stewartii* BACTERIAL WILT OF MAIZE

National Bureau of Agricultural Commodity and Food Standards

Ministry of Agriculture and Cooperatives

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Thailand still faces serious problems in exportıng corn seeds due to the fact that the trading partner countries' complain of infection of *Pantoea stewartii* subsp. *stewartii* which is the pathogen causing maize bacterial wilt and is prohibited in international trade. The corn seed exported from Thailand have been found such bacteria and so destroyed many times which caused negative image and credibility to the country. Currently, Thailand does not have a reliable guideline on the diagnosis of bacterial wilt *Pantoea stewartii* subsp. *stewartii* to gain a full confidence from the trading partner countries. It is therefore, necessary to establish a national agricultural commodity and food standard on the diagnostic protocols for *Pantoea stewartii* subsp. *stewartii* a bacterial wilt of maize for official guidelines for the public and private laboratories working in the plant pest diagnosis. This is to promote the use of phytosanitary measures for facilitating the import and export of corn seeds.

The provisions of this standard are based upon the data derived from the following documents:

1. The project titled on Diagnosis Techniques of *Pantoea stewartii* subsp. *stewartii*, a bacterial wilt of maize conducted by the National Bureau of Agricultural Commodity and Food Standards in collaboration with the Agricultural Biotechnology Centre, Kasetsart University. B.E. 2550 (2007)

2. OEPP/EPPO. 2006. *Pantoea stewartii* subsp. *stewartii* Diagnostics. In Bulletin OEPP/EPPO Bulletin 36.

Remark:
The standard title has been revised from "Thai Agricultural Commodity and Food Standard (TACFS)" to "Thai Agricultural Standard (TAS)" in accordance with the enforcement of the Agricultural Standards Act B.E. 2551 (2008)

NOTIFICATION OF THE NATIONAL COMMITTEE ON AGRICULTURAL COMMODITY AND FOOD STANDARDS SUBJECT: THAI AGRICULTURAL STANDARDS: DIAGNOSTIC PROTOCOLS FOR *Pantoea stewartii* subsp. *stewartii* BACTERIAL WILT OF MAIZE B.E. 2551 (2008)

The resolution of the 1/2551 session of the National Committee on Agricultural Commodity and Food Standards dated 11 August B.E. 2551 (2008) endorsed on the establishment of the Thai Agricultural Commodity and Food Standard entitled Diagnostic Protocols for *Pantoea stewartii* subsp. *stewartii* Bacterial Wilt of Maize. This standard is to promote the effective diagnosis of quarantine plant pests and to be internationally accepted.

By virtue of the Cabinet Resolution on Appointment and Authorization of the National Committee on Agricultural Commodity and Food Standards dated 5PPPPPPPP August B.E.2551 (2008), the Notification on Thai Agricultural Commodity and Food Standard entitled Diagnostic Protocols for *Pantoea stewartii* subsp. *stewartii* Bacterial Wilt of Maize is hereby issued as voluntary standard, the details of which are attached herewith.

Notified on the 14 October B.E. 2551 (2008)

Mr. Somsak Prissana-nanthakul
Minister of Agriculture and Cooperatives
Chairperson of the National Committee on Agricultural Commodity and Food Standards

THAI AGRICULTURAL STANDARD

DIAGNOSTIC PROTOCOLS FOR *Pantoea stewartii* subsp. *stewartii* BACTERIAL WILT OF MAIZE

1 SCOPE

This agricultural commodity and food standard sets diagnostic protocols for *Pantoea stewartii* subsp. *stewartii* which is a bacteria causing wilt of maize. The Standard is to be used as a laboratory diagnostic guide for plant pests in corn seeds which is a part of phytosanitary measures for the export and import of this produce. This standard was developed from the [TACFS 9030-2008] 'Thai Agricultural Commodity and Food Standard Diagnostic Protocols for Regulated Pests '.

2 *Pantoea stewartii* subsp. *stewartii*, a bacterial wilt of maize

2.1 General information and distribution

Pantoea stewartii subsp. *stewartii* is a Gram negative stain, rod-shaped and non-motile bacteria with cell size of 0.4 – 0.8 x 0.9 -2.2 µm. The optimal temperatures for this bacterial growth range between 27 – 30°C, while at the temperature of 8 – 9°C the bacteria grow at the lowest rate and its thermal death point is at 53°C (Pepper, 1967). These bacteria can be transferred through corn seeds and can be distributed to other areas. As reported in the Crop Protection Compendium 2005, the *Pantoea stewartii* subsp. *stewartii* is indigenous of North America, i.e. Canada, Mexico and the United States of America; Central America and the Caribbean, i.e. Costa Rica, Pertorico; and South America, i.e. Bolivia, Brazil, Peru and Guyana; Europe and the Mediterranean, i.e. Austria; and Asia, i.e. India. The primary host of this bacterium is maize, which included of sweet corn, dent, flint, flour, baby corn and popcorn cultivars with sweet corn being the most susceptible. This bacterium is transmitted by corn flea beetle (*Chaetocnema pulicaria* Melsh.) which is the main living host that carries the bacterium across winter and spread in the next maize plantation. Wind is the most effective factor of distribution. Therefore, disease control should be focused on the use of disease-resistant varieties together with the weather forecasting system to assess the survival rate of the insect host which will enable the prediction of disease occurrence in the next crop season. The use of chemicals may not be effective if low disease-resistant varieties are used (Nutter-Jr., *et.al.*, 2002; Nutter-Jr. and Esker, 2003; Kuhar, *et.al.*, 2002).

Further information on biology, distribution, symptoms on host, economic importance of This bacterium is provided in EPPO/CABI (1997, 1998) or Pataky and Ikin (2003).

2.2 Disease symptoms

Pantoea stewartii subps. *stewartii* may be found in corn seeds without visible characteristics symptoms. The symptoms on the stems of maize can be divided into two phases. The first phase; at young seedling stage the disease spreads systematically through the vascular system

starting from the top leaves causing a wilting symptom which later on develops to other parts. The second phase; at maturity stage the leaves show pale-green to yellow (Figure A.2) starting from a small insect-bite spot and expand to longitudinal streaks, with irregular or wavy margins, which are parallel to the veins. These streaks may dry out and turn straw-coloured. Plants that are not killed appear pale-coloured leaves and dead tassels. Cavities may appear close to the soil in the stalk pith of severely infected plants with a large volume of *Pantoea stewartii* subsp. *stewartii.* Sometimes the bacterium may exude in fine droplets or ooze on the husk of the corn cops. It can penetrates deeply in the seeds in chalazal area of the aleuronic layer and between the endosperm cells, but not in the embryo and the seed coat.

The disease may be confused with other leaf blights of maize;

(1) Northern corn leaf blight[1/]; is caused by a fungus *Setosphaeria turcica* (*Bipoloris turcica*), with spindle-shaped, greyish-green to tan spots.
(2) Southern corn leaf blight[1/]; is caused by a fungus *Cochiliobolus heterostrophus*, with well defined tan to brown spots.
(3) Leaf spot; is caused by a fungal Cochiliobolus carbonum; with light to dark brown spots.
(4) Leaf blight and vascular wilt; is caused by a bacterium *Pantoea agglomerans*, pale-green leaves with long narrow stripes or spots, with wavy reddish-brown edges (Morales-Valenzuela, *et.al*., 2007).

Moreover, the disease symptoms may be confused with water and some nutrients deficiencies, as well as the insect bites.

3 TAXONOMY

Name: *Pantoea stewartii* subsp. *stewartii* (Smith, 1898) Margaret, *et. al*., 1993.

Other names: *Erwinia stewartii* (Smith, 1898) Dye, 1963.
Xanthomonas stewartii (Smith, 1898) Dowson, 1939.

Common names; Maize wilt
Stewart’s diease, Stewart’s wilt, Stewart’s bacterial wilt, Bacterial wilt and Bacterial wilt of maize (English)
Maladie de Stewart and flétrissement bactérien (French)

Taxonomy of the Bacterium

Domain: Bacteria
Phylum: Proteobacteria
Class: Y-Proteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Pantoea*
Species: *stewartii*
EPPO code: ERWIST
Phytosanitary categorization: EPPO A2 list no. 54; EU AnnexII/A1

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[1] No perfect stage is yet found in Thailand

4 DETECTION

4.1 Preparation for the extraction of *Pantoea stewartii* subsp. *stewartii* from stems or leaves of maize

Samples are derived from infected leaves, stems or different parts of maize that show symptoms. The infected and non-infected parts are separated, surface cleansed and dried with paper. Then cut the samples into small pieces for disease detection. If the polymerase chain reaction or PCR method is practiced, the cut samples are soaked in distilled water, while the bacterial extraction through medium method requires the samples to be soaked in an appropriate volume of calcium chloride solution 0.85% or sterile phosphate-buffered saline. Both methods require a 30-minute centrifuge before the clear solution is extracted for testing by different methods.

4.2 Preparation for the extraction of *Pantoea stewartii* subsp. *stewartii* from seeds of maize

4.2.1 ELISA Method (Enzyme-Linked Immunosorbent Assay)

Four hundred seeds of maize are drawn as samples following the seed sampling standards of the International Seed Testing Association- ISTA). The samples are divided into 4 piles, each contains 100 seeds. Soak the seeds overnight at the rate of 100 ml of water per 100 seeds at the temperature of 4°C. The clear soaking liquid of 30 ml is then sent for disease detection using PCR method. The mixture of the remaining 70 ml liquid and the soaked seeds is added with 7 ml of 10X General Extraction Buffer (GEB) to bring its final concentration to 1X (the 1X GEB solution contains sodium sulfite (anhydrous) 1.3 grams, Polyvinylpyrrolidone (PVP) MW 24-40,000 20.0 grams, Sodium azide 0.2 gram, powdered egg (chicken) albumin, Grade II 2.0 grams and Tween-20 20.0 grams in the 1X PBST soluble at 1,000 ml pH of 7.4 at the temperature of 4°C). The soaked seeds are blended, filtered with thin white cloth to separate the liquid for ELISA test. In case there are several batches of sub-samples, the blender needs to be cleaned with water and washing detergent before the next use.

4.2.2 PCR Method (Polymerase Chain Reaction)

The test is performed by taking 30 ml of soaking liquid from 4.2.1 above to be filtered with No.1 Whatman®, followed by a filtration with bacterial filter mixed cellulose ester membrane of 0.45 µm porous sizes. Each bacterial filter is placed in a Petri dish filled with 3 ml of water. The Petri dish is later centrifuged at the rate of 70 rev. / minutes for 30 minutes. Transfer 1 ml of the soaking liquid in the Petri dish for a stain centrifuge at 8,000 *g* for 5 minutes. The stain is the dissolved with 50 µl of water, and the solution is used for PCR test. This solution can be kept at 4°C for other testing methods.

4.3 Detection of *Pantoea stewartii* subsp. *stewartii*

4.3.1 ELISA Method

The solution extracted from seeds in 4.2.1 can be tested with an ELISA’s instant testing set called Agdia® where the recommendations are provided. If the testing results are positive, the solution should also be tested with other testing methods.

The advantages of this method are; easy to repeat, low cost, able to detect bacteria when the amount is over 10^5 cell/ml and can detect the contamination of bacteria in seed, plant and

insect samples. However, its limitations is unable to detect the living condition of the bacteria, positive value needs to be 3 times higher than that of the negative control, i.e. the solution extracted from seeds or plant parts. Any value that is higher but not more than 3 times is treated as elevated value and the testing needs to be repeated, (Sutula, *et.al*., 1986). Moreover, this instant testing set can yield positive results of other close-relation types of bacteria (Juthathep, *et.al*., 2007).

4.3.2 PCR Method

The stain solution from 4.2.2 is used for PCR test, using specific paired primers *hrpS* gene (Coplin and Majerczak, 2002). This starts with mixing 2 µl of stain solution with reaction mixture which contains a solution of the final concentration as below;

1X PCR buffer
0.20 µm dNTPs
0.25 mM $MgCl_2$
*0.20 µm primer HRP1d (5' GCACTCATTCCGACCAC 3')
*0.20 µm primer HRP3c (5' GCGGCATACCTAACTCC 3'),
**0.20 µm primer fd2 (5' CCGAATTCGTCGACAACAGAGTTTGATC- ATGGCTCAG 3')
**0.20 µm primer rp1 (5' CCCGGGATCCAAGCTTACGGTTACCTTG- TTACGACTT 3')
Taq DNA polymerase 0.3 unit

the sample solution of 2 µl in the total solution reaction of 15 µl.
(* = paired primers per *hrpS* gene, ** = paired primers per 16S rDNA gene of the Enteric bacterial group).

Reaction to increase the DNA parts can follow the steps below;

Step 1: the sample is incubated at 94°C for 120 s.
Step 2: the sample is incubated at 94°C for 20 s.
Step 3: the sample is incubated at 54°C for 15 s.
Step 4: the sample is incubated at 72°C for 90 s.
Then repeat the reaction from Step 2 to step 4 for 24 times.
Step 5: the sample is incubated at 72°C for 5 s.

The DNA is then detected by Agarose Gel Electrophoresis technique. DNA sizes are separated on 0.8% agarose gel in 0.5X TBE at an electric current of 100 Volt for 40 minutes. The standard DNA is used as a control.

DNA bands are detected by staining the gel with 0.5 µg/ml ethidium bromide for 10 minutes and rinse with clean water or 0.5X TBE for 10 minutes. The DNA bands are then detected under the UV ray. This testing technique requires a control reaction tube which does not produce any DNA band since it contains water only, but must yield DNA band of 1,500 bp for the 16s rDNA, which is the indicative control showing the Polymerase Chain Reaction. This specific paired polymers for 16s rDNA gene can only yield a reaction on the Enteric bacteria group and must yield a DNA band of 900 bp for the *hrpS* gene. Moreover, the polymers for cpsDE gene and 16s-23s ITS gene can also be used to detect the bacteria as well (Coplin and Majerezak, 2002). If the testing results are positive, other testing methods should also be applied.

The advantages of the PCR techniques are; a sensitive technique to detect *Pantoea stewartii* subsp. *stewartii*, purity can be obtained at the level of 20 cells, and able to detect as high as 200 bacterial cells per reaction from plant cells. However, there are some limitations; it is unable to detect the living condition of the bacteria, unable to detect some mutated bacteria, such as *Pantoea stewartii* subsp. *stewartii* strain DC116, which is not an important strain. Juthathep, *et.al.* (2007) found that this polymer reaction could yield positive reaction to close-relation bacteria, such as *Pantoea agglomerans* in Thailand.

5 IDENTIFICATION

5.1 Isolation of pure *Pantoea stewartii* subsp. *stewartii*

The solution prepared in 4.2.1 or 4.2.2 that yields positive results is diluted by serial dilution for 10 times with water or 0.85% sodium chloride or sterile phosphate buffer. 100 µl of each dilution is spread over onto the medium NA or LB, or King's B in agar plates supplemented with 5% sodium chloride and 200 mg/l cycloheximide. The plates are incubated at 30ºC, and examined after 3-4 days for colonies of *Pantoea stewartii* subsp. *stewartii*. Colonies are yellow, shiny, flat or convex, translucent and with the surface sunk like a volcano (Figure A.3 (c)). Dilute the targeted colonies with 500 µl of water, mix them well and spread on the same type of medium to isolation.

5.2 Identification of *Pantoea stewartii* subsp. *stewartii*

5.2.1 Specific property test

The pure culture of bacteria are checked for motility, Gram-staining, etc. and compare between the properties of *Pantoea stewartii* subsp. *stewartii* with other similar species, see Table 1. If the results are the same as *Pantoea stewartii* subsp. *stewartii*, they should be confirmed by PCR test, Biolog, PFGE assay, or REP-PCR, and pathogenicity test.

Table 1 Comparison of the properties of *Pantoea stewartii subsp. stewartii* and distinction from similar species.

Test	*Pantoea stewartii* subsp. *stewartii*	*Pantoea stewartii* subsp. *ndologenes*	*Pantoea agglomerans*	*Pantoea ananas*
Motility	Non-motile	Motile (or non-motile)	Motile	Motile
Gram staining	Negative	Negative	Negative	Negative
Cytochrome C oxidase	Negative	Negative	Negative	Negative
Catalase test	Positive	Positive	Positive	Positive
Esculin hydrolysis	Negative	Positive	Positive	Positive
Acetoin production	Negative	Positive	Positive	Positive
Indole production	Negative	Positive	Negative	Positive
Nitrate reduction	Negative	Negative	Positive	Viriable
Growth on *cis* – aconitate	Negative	Positive	Negative	Positive
Acid production from:				
Maltose	Negative	Positive	Positive	Positive
Arbutin	Negative	Positive	Positive	Positive
Salicin	Negative	Positive	Positive	Positive
Raffinose	Positive	Positive	Negative	Positive
Cellobiose	Negative	Positive	Variation	Positive
Arabitol	Negative	Positive	Variation	Positive

Negative means yield the negative results
Positive means yield the positive results
Variation means results can be either positive or negative
Source: Mergaert, *et.al*., 1993.

5.2.2 PCR Method

This method is to test the pure culture of *Pantoea stewartii* subsp. *stewartii* separated by paired primers and undergone reaction under the same process as PCR test in 4.3.2.

5.2.3 Biolog Method

This test is performed to test pure culture of *Pantoea stewartii* subsp. *stewartii* using Biolog GN2 (Biolog® Inc., Hayward, CA, USA) following the manufacturer's instructions.

5.2.4 Genetic fingerprinting and protein

Genomic DNA are compared grouped using PFGE assay (Pulse Field Gel Electrophoresis) (Zhang and Geider, 1997), or by REP-PCR technique (Repetitive Extragenic Palendromic – PCR) following the experiment of Louws, *et.al*. (1994), or compared proteins of the whole cell with SDS-PAGE (Sodium Sulfate Polyacrylamide Gel Electrophoresis) following the experiment of Margaert, *et.al*. (1983).

5.2.5 Pathogenicity test

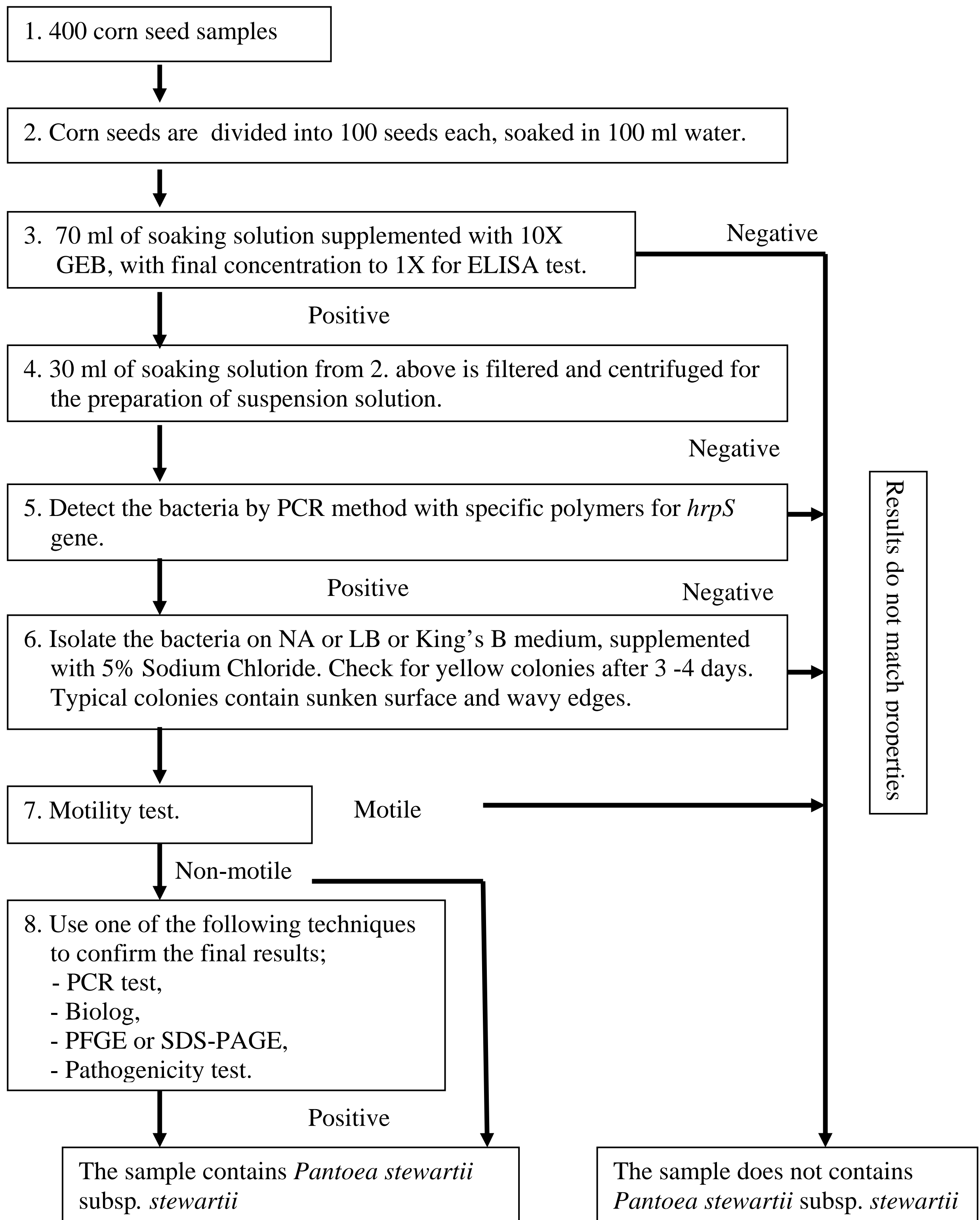
The pathogenicity test is carried out by inoculating the isolated pure culture of *Pantoea stewartii* subsp. *stewartii* on the 5 – 10 days old stem sweet corn of *Insee* 2 cultivar. The plants are inoculated by syringe with 108 – 109 cells / ml of bacterial suspension into the back of the leaves (infiltration method), or by whorl inoculation method without making scar on the leaves. After inoculation, the plants are kept in a chamber where the relative Humidity (RH) is over 90% at 25 – 28ºC for 3 days. If it is *Pantoea stewartii* subsp. *stewartii,* the first disease symptom should appear, i.e. water soaking at the injection spots, or the tip rots or watery spots on the leaf edges if the whorl inoculation is used.

Bacteria of similar species are; *Pantoea agglomerans* which can create water soaking spots or wilt as *Pantoea stewartii* subsp. *stewartii*. When tested the flow of bacterial flow under a microscope, they perform similar patterns. Therefore, other bacterial detecting methods are also needed, such as biolog, or other methods as shown in Table 1.

5.3 Reference strain

(1) *Pantoea stewartii* subsp. *stewartii*, such as ATCC 8199; CFBP 2349; ICMP 257; LMG 2715; NCPB 2295.
(2) *Pantoea stewartii* subsp. *indologenes,* such as ICMP 77; LMG 2632; NCPPB 2280.
(3) *Pantoea agglomerans* (herbicola), such as ATCC 27155; ICMP 12534; LMG 1286 D

Figure 1 Diagnosis of *Pantoea stewartii* subsp. *stewartii*, in corn seeds



6 REPORTING AND DOCUMENTATION

Data to be collected and stored include the followings;

(1) Scientific name of the pest identified.
(2) Code or reference number of the samples for traceability.
(3) Nature of the infected material and their scientific names of host .
(4) Origin (including geographical position, if known) of the infected material and location of interception or detection.
(5) Description of signs or symptoms including photographs where relevant or their absence.
(6) Methods including the controls, used in the diagnosis and the results obtained with each method.
(7) For Morphological or Morphometric methods, measurements, drawings or photograph of the diagnostic features symptoms (where relavant) and, if applicable, an indication of the developmental stage(s).
(8) For biochemical and molecular methods, documentation of test results such as photographs of diagnostic gels or ELISA printouts of results on which the diagnosis was based.
(9) Where appropriate, the magnitude of any infection (how many individual pests found, how much damaged tissue).
(10) The name of the laboratory and, where appropriate, the name of the person(s) responsible for and who performed the diagnosis.
(11) Date of collection of the sample, and of detection and identification of the pest.
State of the pest, alive or dead, or viability of its development stages. Other evidences such as, culture(s) of the pest, nucleic acid of the pest, preserved/mounted specimens or test materials (e.g. photograph of gels, ELISA plate printouts results should be retained in particular in case of non-compliance (ISPM No.13: Guidelines for the notification of non-compliance and emergency action) and where pests are found for the first time the (ISPM No. 17: Pest reporting). Additional items may be required under other ISPM No. 8 (Determination of pest status in an area)).
(13) The period for which records should be kept depends on the purpose for which a diagnosis is made. In cases where other contracting parties may be adversely affected by the results of the diagnosis, records and evidence of the results of the diagnosis should be retained for at least one year.

7 FURTHER INFORMATION

Further information can be obtained from/:

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ANNEX A

Illustration of the diagnosis of *Pantoea stewartii* subsp. *stewartii*

(Refer to points 2.2, 4.3.2, 5.1 and 5.2.1)

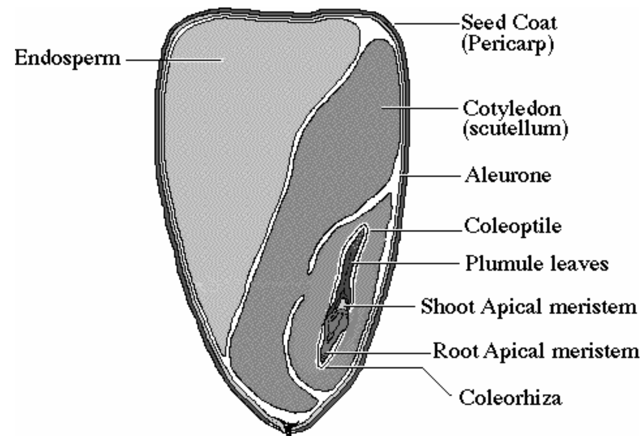

Figure A.1: Structure of Corn Kernel
Source: http://home.earthlink.net/~dayvdanls/cornkernel.gif

Figure A.2: Symptoms of maize wilt caused by *Pantoea stewartii* subsp. *stewartii*
Source: http://www.rec.udel.edu/Update06/Volume14,Issue11_files/image002.jpg
http://www.cropsci.uiuc.edu/faculty/pataky/pubs/bytopic/images/stew2.jpg

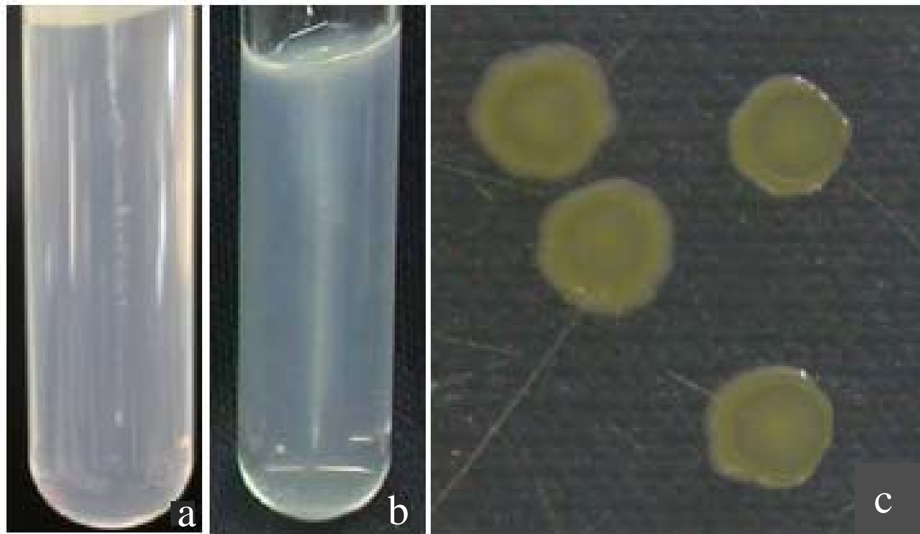

Figure A.3: Bacterial motility test using motility test medium

(a) *Pantoea stewartii* subsp. *stewartii* LMG2715 (non-motile)
(b) *Pantoea agglomerans* (motile)
(c) Colonies of *Pantoea stewartii* subsp. *stewartii* LMG2715 on 3-day old NA medium

Source: Juthathep Watcharachaiyakoop

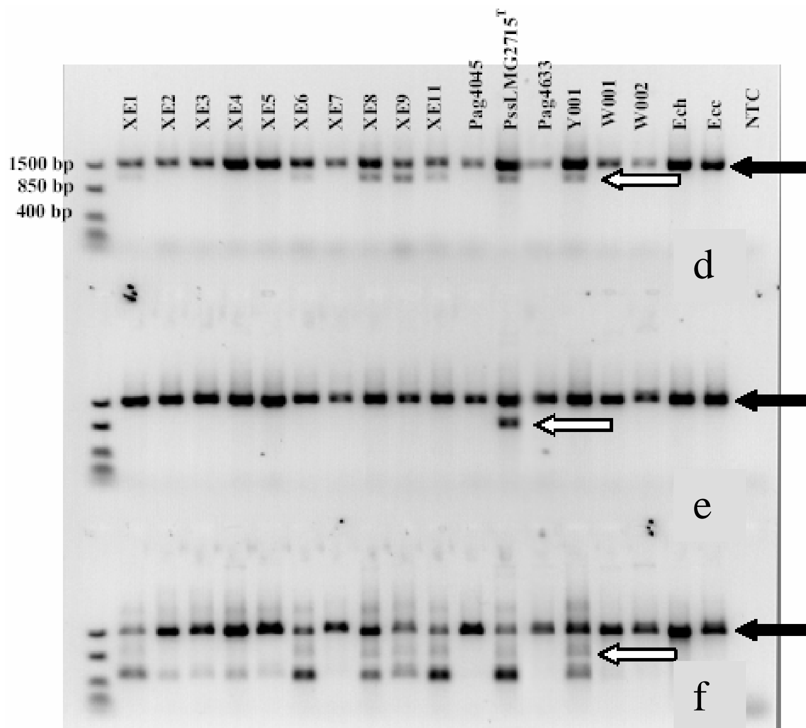

Figure A.4: DNA band of PCR product resulted from bacterial test, separated from maize kernel and *Pantoea stewartii* subsp. *stewartii.* The product was synthesized by specify polymer per cpsDE gene of 1.1 kb

(d) 0.9 kb of *hrpS* gene
(e) and 16s – 23s rRNA/ITS of 0.9 kb size
(f) White arrow positions refer to genes' DNA bands, black arrow positions are DNA of genes of 16s rDNA of 1.5 kb used as control of PCR synthesis.

Source: Juthathep, 2008

ANNEX B

UNITS

Units and symbols used in this Standard are based on the International System of Units or *Le Systéme International d' Unités* (SI) which is widely acceptable;

Item	Name of Units	Symbols
Mass	gram	g
	microgram	µg
	milligram	mg
	kilogram	Kg
Volume	litre	l
	micro litre	µl
	milli litre	ml
Length	centimetre	cm
	micrometre	µm
Temperature	degree Celsius	ºC
Time	second	s
Concentration of substances	milligram per litre	mg / l
	microgram per milli litre	µg / ml
Number of cells	cell per milli litre	cell / ml
Gravity value	gravity	*g*