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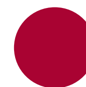

Sampling-detection procedures

bacterial leaf spot pathogen of tomato. Technical bulletin

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Sampling - detection Procedures Bacterial leaf spot of tomato

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Cover pages: bacterial leaf spot symptoms caused by *Xanthomonas* sp. and membrane-based sampling procedures (front cover); farmer harvesting tomato fruits ready for the market (back cover)

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Sampling-detection procedures: bacterial leaf spot pathogen of tomato

TECHNICAL BULLETIN

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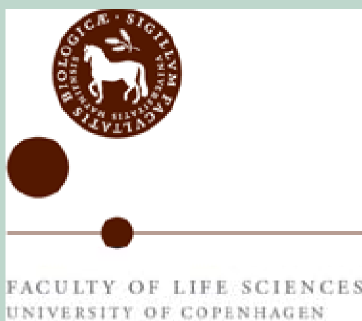

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INTRODUCTION

Bacterial leaf spot (BLS) caused by *Xanthomonas* spp. is a serious disease and a major constraint in tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annum* L.) production worldwide. Infected tomato and pepper plants show different type of leaf spot and fruit symptoms, yield reduction and poor fruit quality.

Tomato is an important cash crop and constitutes 39 % of the total vegetable consumption in Tanzania, providing vitamins and minerals to the diet of the population. Although tomato has a high demand for fresh markets and canning, the national average yield remains low. Factors contributing to the low yield include insect pests and diseases, of which bacterial diseases account for 45 % of yield losses. *Xanthomonas euvesicatoria, X. vesicatoria, X. perforans* and *X. gardneri* have been reported as the main causal agents of BLS. Other organisms such as *X. campestris* pv. *raphani* (Xcr) and *X. arboricola* have also been reported to be pathogenic in tomato and pepper.

In the period 2008-2010, leaf spot symptoms were observed on tomato plants growing in Tanzania (**Fig. 1)**. Symptoms observed were dark, circular to irregular, water-soaked spots surrounded by chlorotic halos (**Fig. 2**). Samples of leaves and tomato fruits were collected in 2010 from affected field plants and used for tissue printing on FTA/Whatman membranes were taken to a central laboratory for the diagnosis of the causal agents of the disease symptoms.

The tissue-print samples supported a relatively quick and acurate PCR-based assay. All reported xanthomonads from tomato with the exception of *X.c.* pv. *raphani* were recovered on nutrient agar medium from tissue prints made from infected plants and/or infected seeds collected from the main growing production areas in Tanzania.

It is expected that this puplication will be for the benefit of farmers, extension agents and control agencies which have limited access to information on sampling and diagnosis of the BLS disease which is often difficult to establish by field inspections alone. Infected plant samples thus submitted in the form of tissue-prints made onto FTA or Whatman paper membranes can be sent in a safe manner to a central diagnostic laboratory for PCR assays or for isolation of the pathogen(s).

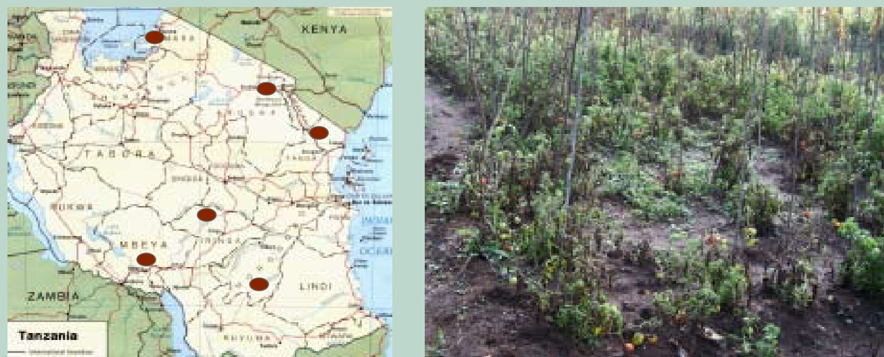

Fig. 1. Areas of survey (left) and tomato field plants severely affected by the bacterial leaf spot disease of tomato in Tanzania, 2010

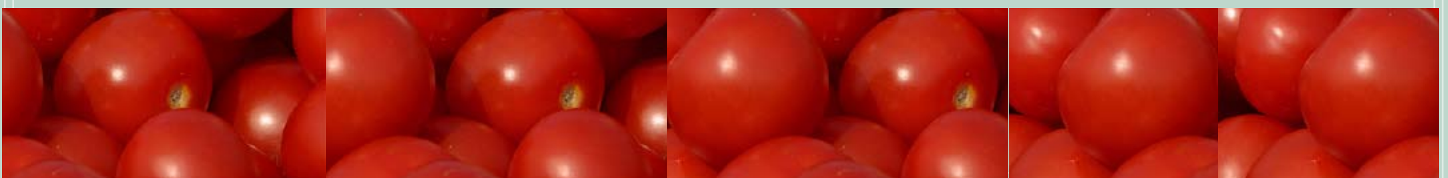

Fig. 2. Symptoms of bacterial leaf spot disease of tomato

Fig. 3

Fig. 4

SAMPLING PROCEDURE

1. Label FTA plant card and Whatman paper strips

2. Select leaves/fruits sections showing BLS symptoms
   Collect tissue in plastic bags avoiding cross-contamination between samples

3. Macerate tissue (3g) collected in plastic bag by hand (**Fig. 3**) or in a morter with pestle (1g tissue/3 ml sterile distilled water)

4. Cut the corner of the plastic bag and place plant sap samples onto FTA plant card or Whatman paper strips (**Fig.4**); samples 50-75µl from morter macerate are added to the membranes

5. Allow samples to air-dry (15-30min): *Keep samples without direct exposure to sun; avoid cross contamination of samples (samples can be stored at room temperature in paper or plastic bags)*

« Sample information »

Company name/ Your name: --

Address: ---
Phone #: -------------------------------------Fax #: -----------------
Name of host:--
Variety or cultivar:--
Location of plot: --
Sample #---
Date:-------------------------- Signature-----------------------------

Diagnostic Laboratory in Tanzania:

African Seed Health Centre (AfSHC)
Sokoine University of Agriculture
P.O. Box 3005, Morogoro, Tanzania
Phone: +255 232 600 573; E-mail: rmabagala@yahoo.com;
rbm@afshc.or.tz; Fax: +255 232 600 574; www.afshc.or.tz

Shipment of samples to the diagnostic laboratory:

Molecular level tests confirm the presence or absence of the pathogen in the collected sample

1. Ship to us the collected samples for delivery within 1-2 days. Include a cover letter with your contact person.

2. For PCR assays, please, submit tissue-print captured onto FTA or Whatman paper membranes which can be provided by the near extension service officer in the region after agreement with the central diagnostic laboratory.

3. To avoid any delays, and if requested submit enough plant material to complete the isolation of the causal agent with the requested information with the sample.

4. Alternatively those with lab facilites for isolation of the pathogens can send samples in pure culture in agar slants/stubs in test tubes or on agar plates properly sealed and labelled.

PCR DETECTION for *XANTHOMONAS* (Mbega, 2011)*

1. Punch 2mm discs from FTA plant cards or Whatman paper strips (**Fig. 5**)

2. Wash FTA paper discs prior adding to PCR mix (follow instructions of the manufacturer); Whatman paper strips discs can be added directly without washing into PCR master mix

3. Conduct PCR using genus *Xanthomonas* primers (Adriko et al., 2011)**: *gum*D-fw 5`/*gumD*-rv 5`; a reference strain of a tomato *Xanthomonas* spp. serves as positive control; genus primers can be multi plexed with mitochondrial 26S rRNA host primers (m26S F4/R3) that may serve as internal controls of the PCR reactions

4. ***Xanthomonas* - PCR:** conduct amplifications with final volume of 25µl containing 1.5µl mM of $MgCl_2$, 5µl buffer 5x, 4µl of 1.25 mM dNTP, 0.25µl of 40pM of each primer and 0.25µl of 0.25U of *Taq* polymerase(Promega™, Madison, Winscosin, USA) and12.75µl sterile distilled water

5. Amplification reactions are run for 40 cycles, each consisting of 30s at 95^0C, 15s at 65°C, and 15s at 72^0C, with an initial denaturation of 3 min at 95°C and a final extension of 3min at 72^0C

6. Take samples of 9µl from each amplified PCR product and run on a 1.5% agarose gel stained with 12µl of 0.5µg.ml^{-1} ethidium bromide at 50V for 40min and visualised on a UV transiluminator

7. An expected amplicon of 402bp is an indication of a positive recognition of xanthomonads from the submitted sample; in multiplex PCR reactions a second band of 594bp can be seen from the internal control primers for the reactions. The second band can only be observed with FTA plant cards with DNA template obtained from morter macerated plant extracts (**Fig. 6**).

Fig. 5. Discs of 2mm taken with a Harris Micro Punch™

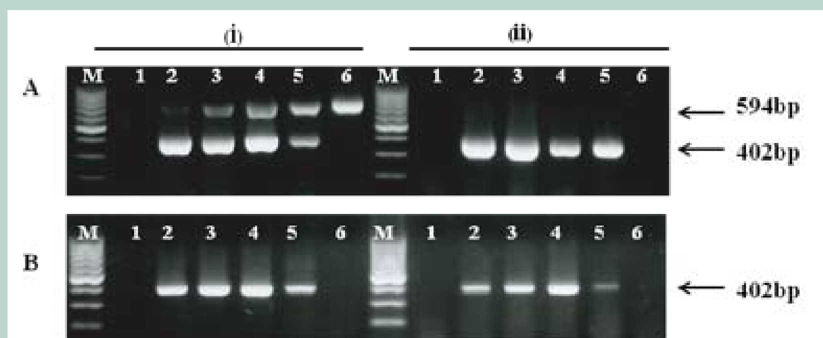

Fig. 6. Detection of BLS xanthomonads from FTA plant card (A) and Whatman paper strips (B) in multiplex PCR using genus *Xanthomonas* specific (Xan7) primers and mito-chondrial 26S rRNA primers. (i) tissue prints prepared by placing 50-75µl samples of plant extract from tissue macerated in a mortar on the paper membranes; (ii) samples are crushed with fingers while in plastic sheet and extracts pressed through a small hole made with a sterile needle onto paper membranes. Molecular weight marker (lane M), PCR master mix (lane 2); Lanes 2-6, leaf prints originated from tomato plants infected with strains of *X. eu-vesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422, *X. perforans* NCPPB 4321, *X. gardneri* NCPPB 88, respectively, and healthy plant control (lane 6)

*E.R. Mbega, 2011. Detection, Characterisation and Control of *Xanthomonas* spp., the Causal Agents of Bacterial Leaf Spot of Tomato in Tanzania. PhD Thesis. Dep. Agric. & Ecology, Faculty of Life Sciences, University of Copenhagen, Denmark.
**J. Adriko. 2011. Molecular Detection of *Xanthomonas* at the Genus and Pathovar Levels.PhD Thesis. Dep. Agric. & Ecology, Faculty of Life Sciences, University of Copenhagen, Denmark.

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