DANIEL HENRIQUE RIBEIRO

MONITORING *Clavibacter michiganensis* SUBSP. *michiganensis* GFP – MARKED STRAIN INOCULATED IN TOMATO SEEDS

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de pós-graduação em Agronomia/Fitopatologia, para a obtenção do título de Doutor.

Orientador:

Dr. Ricardo Magela de Souza

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2015

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GENERAL ABSTRACT

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is an economically-important seed-borne phytobacteria that causes bacterial canker and bacterial wilt, the most important seed-borne disease of tomato. For studies on colonization of tomato seeds, seed treatments, seed transmission and for evaluation of diagnostic methods with Cmm, there is a need for seed that is internally infected with Cmm, how it is observed in natural infections. In this study, a procedure was developed to generate tomato seed (internally-) infected with Cmm. These seeds can be used to evaluate methods for detection of Cmm in seed extracts, to study the effect of seed treatments and to study transmission from seed to seedlings. Seeds were subsequently wounded by scarification, softened by incubation on wetted blotting paper, and inoculated with a spontaneous rifampicin resistant Cmm strain suspension by vacuum-infiltration. A GFP-tagged Cmm strain was used to visualize Cmm *in planta*. The population dynamics of Cmm on or in (disinfected) seeds and seedlings were studied by plating, a non-invasive monitoring system for GFP-tagged bacteria in plants (PathoScreen), Confocal laser scanning microscopy (CLSM), Epifluorescence stereomicroscopy (ESM) and a TaqMan assay. Also, dead and viable seeds were used to observe the Cmm behavior on and in different seed tissues. To enhance growth of the Cmm in the seeds, they were incubated on wetted blotting paper or on a Cmm-selective medium/broth. Directly after inoculation, a few single Cmm cells could be spotted into the seeds and incubation of seeds resulted in a strong increase of Cmm populations. The increase of the bacteria into the seed was confirmed with the monitoring of the GFP signal by the PathoScreen analyzes. Transected and undivided seeds analyzed with CSLM and ESM indicated that during incubation, Cmm colonized trichome hairs, the outer testa layers, the endosperm and embryo. The GFP signal could also be observed by epifluorescence stereomicroscopy in germinated seeds on the seed coat, cotyledons, stem and roots. Colonization of xylem vessels in stems and roots of seedlings was observed with confocal laser scanning microscopy, indicating systemic infections of germinated seeds. Previous incubation of infected seeds in semi-selective media improved the detection of Cmm by a TaqMan assay. This enhancement was obtained with the increasing of low population densities of Cmm in tomato seeds.

Keywords: bacterial canker, confocal laser scanning microscopy, epifluorescence stereomicroscopy, green fluorescent protein, seedlings, TaqMan assay, qPCR.

RESUMO GERAL

Clavibacter michiganensis subsp. *michiganensis* (Cmm) é a bactéria causadora da murcha e cancro bacteriano do tomateiro, a mais importante doença transmitida por sementes. Para estudos sobre colonização, transmissão e avaliação de métodos de diagnose de Cmm em sementes, existe a necessidade de infecções internas, como qual se é observado em infecções que ocorrem naturalmente. Neste estudo, um procedimento foi desenvolvido para gerar sementes de tomate internamente infectadas com Cmm. Estas sementes podem ser utilizadas em avaliações de métodos para detecção em extratos de sementes, para estudo do efeito do tratamento de sementes e para o estudo na transmissão de sementes para mudas. Para tal, sementes de tomate foram feridas por escarificação, amolecidas pela incubação em blotters umedecidos e inoculadas com a suspensão de um isolado de Cmm naturalmente resistente a Rifampicina por infiltração a vácuo. Um isolado de Cmm marcado com GFP foi utilizado para a visualização de Cmm *in planta*. A dinâmica populacional (interna e externa) de Cmm em sementes e mudas de tomate foi estudada por plaqueamento, por um sistema de monitoramento não invasivo para bactérias marcadas com GFP em planta (PathoScreen), por Microscopia de escaneamento com laser confocal (CLSM), Microscopia de epifluorescência (ESM) e um ensaio com TaqMan. Sementes mortas e viáveis foram utilizadas para a observação do comportamento de Cmm em diferentes tecidos. Para estimular o crescimento de Cmm nas sementes, estas foram incubadas em blotters umedecidos ou em um meio/solução nutriente semi-seletivo para Cmm. Imediatamente após a inoculação, algumas células de Cmm puderam ser observadas dentro das sementes e a incubação dessas sementes resultou em um grande aumento desta população. Este aumento da densidade populacional dentro da semente foi confirmado com o monitoramento do sinal de GFP por análises de PathoScreen. Sementes inteiras e cortadas transversalmente analisadas com CLSM e ESM indicaram que durante a incubação, Cmm coloniza os tricomas, as camadas exteriores do tegumento, o endosperma e o embrião. O sinal de GFP também pôde ser observado por ESM na capa de sementes germinadas, cotilédones, caule e raiz. Colonização dos vasos do xilema no caule e raiz das mudas foi observada com CLSM, indicando infecção sistêmica de sementes germinadas. A prévia incubação de sementes infectadas em um meio semi-seletivo aperfeiçoou a detecção de Cmm por TaqMan. Este enriquecimento foi obtido pelo aumento da baixa densidade populacional de Cmm nas sementes de tomate.

Palavras – chave: Cancro bacteriano, Microscopia de escaneamento com laser confocal, Microscopia de epifluorescência, Proteína verde fluorescente, Mudas, Ensaio TaqMan, PCR em tempo real.

SUMÁRIO

TÍTULO: Interactions Monitoring of *Clavibacter michiganensis* subsp. *michiganensis* in Tomato Seeds and Seedlings with a GFP – Marked Strain

FIRST PART

1 – INTRODUCTION

Tomato (*Solanum lycospersicum* L. [syn. *Lycopersicon esculentum* Mill.]) is one of the most important vegetable crops cultivated worldwide. In 2013, the production of tomato in Brazil reached 4,187,646 tonnes, with a yield of 668,024.63 hg/ha. Occupying a place between the 20 more important commodities in Brazil, national tomato consumption reaches 4,012,163 tonnes per year and more than 20 kg per capita. China ranks first in tomato production in the World, with more than 50,000,000 tonnes per year, followed by India (17,500,000 tonnes per year), Unites States of America (13,206,950 tonnes per year) and Brazil occupies the 9th position in this rank (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2013). Tomato plants are stricken with several diseases and among these, bacterial diseases can cause several economic and nutritional losses, due to severe symptoms like plant wilting and death, reduced photosynthetic capacity due to foliar lesions, fruit lesions (field) or netting (greenhouse), and reduced fruit set and size (AMERICAN SEED TRADE ASSOCIATION - ASTA, 2015).

Clavibacter michiganensis subsp. *michiganensis* (Smith) Davis et al. (1984) (Cmm), is a seed-borne pathogen causing bacterial canker and wilt in tomato and it is considered one of the most destructive bacterial disease in this crop (EICHENLAUB; GATERMANN, 2011). For this reason, in the European Union (EU) and in many other countries Cmm is a quarantine pathogen. It was first reported by Erwin Frink Smith at the beginning of the twentieth century in

Michigan (USA), and currently it is present worldwide (EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION - EPPO, 2005). Its movement over long distances is facilitated by traded seeds, which explains its distribution throughout all of the tomato-growing regions of the world, but its spread differs widely among countries. However, it can also survive in plant debris and on volunteer plants or alternative hosts that can act as local sources of inoculums (GLEASON; GITAITIS; RICKER, 1993). This bacterium is transported to new crops by contaminated seeds and transplants, which is resulted from asymptomatic infections on late stages of plant development, but the pathway used by the bacteria to move from the seed to infect the seedlings is not fully understood (XU et al., 2010). To try to elucidate this pathway, Cmm strains were previously transformed with plasmids containing the gene for expression of fluorescent proteins, like eGFP, to follow the movement of Cmm *in situ* (CHALUPOWICZ et al., 2012; LELIS et al., 2013).

In several studies, bioluminescent images, obtained with Cmm strains capable of *lux* genes set expression, were also used to follow the behave of Cmm on infections on tomato plants. This bioluminescent technique provides sensitive detection levels, capacity of monitoring the internalization and distribution of bacteria in hosts, have also been used to track bacterial movement and distribution in host plants, as well as to assess host susceptibility quantitatively (FUKUI et al., 1996; XU et al., 2010, 2011). However, this methodology depends on the enzyme kinetics of cellular uptake of substrates in cell metabolism and cannot be used to follow single bacterial cells or detect them in damaged tissue or dead cells, and sometimes, problems with auto-fluorescent tissues are involved (KOHLMEIER et al., 2007). To prevent the limitations of this technique, the utilization of eGFP as bio-reporter to investigate the colonization patterns and movement of Cmm in infected tomato plants it is an

alternative technique, mainly because eGFP gene set it is already successfully inserted and stably expressed on Cmm and does not affect the pathogen virulence process (CHALUPOWICZ et al., 2012; LELIS et al., 2013).

Studies involving gram positive bacteria and molecular tools of fluorescent reporter genes expression are just a few if comparing to those with gram negative bacteria (CHALUPOWICZ et al., 2012). In this study, a natural rifampicin resistant strain transformed to express eGFP was used to track back the bacteria from artificially infected seeds to seedlings using microscope techniques, such confocal laser scanning microscopy (CSLM) and epifluorescence stereomicroscopy (ESM), providing knowledge about the Cmm behave on/in the seeds and seedlings.

The main source of plant to plant contamination is when Cmm reaches new plants by water splash in rain or irrigation, leaf to leaf contact, wind, insects, contaminated equipment, infected plant residues in soil, symptomless *Solanum* spp., by grafting, via nutrient solution in hydroponically grown tomatoes and others routes and moves epiphytically through the plant parts finding natural openings, like hydathodes and stomata, and wounds (CARLTON; BRAUN; GLEASON, 1998; DE LÉON et al., 2011). After penetrating the plant, the bacteria, as a good endophyte (CARLTON; BRAUN; GLEASON, 1998; GATERMANN et al., 2003), will colonize and move through the plant by biofilm-like structures formed from multiplication in the xylem vessels of the large marginal fimbriate veins, then are distributed to the leaf margins, causing marginal necrosis. After the colonization, the bacterial population leads to a systemic infection characterized by unilateral leaf wilting and canker development on the tomato stem that eventually culminates in plant death (CARLTON; BRAUN; GLEASON, 1998; CHALUPOWICZ et al., 2012; GATERMANN et al., 2003). On the field and greenhouses, yield losses are characterized by wilting and death of tomato plants, reduced photosynthetic

capacity, fruit lesions and reduced fruit set and size. Serious epidemics can cause up to 80% of yield losses (MILLER; ROWE; RIEDEL, 1996).

Despites the fact that internal seed contaminations may be considered low, but its present (EPPO, 2005), the seed infection probably happens through the calyx scar, vascular bundle of the fruit, glandular and nonglandular trichomes or fruit pericarp (TANCOS et al., 2013; UEMATSU; FUJI; OHATA, 1977). It is reported that the amount of Cmm in infected seeds usually averages between 10^2 and 10^4 cfu per seed and this population can be found in the seed coat or in internally infections (EPPO, 2013).

This process of internally-infect seeds acquired for this kind of plant pathogens allows them to survive from seed treatment, colonizing the radicle during germination and attaining global distribution (TANCOS et al., 2013) due the fact that most of the seeds are considered health after a superficial disinfection. However, for Cmm, one to five contaminated seeds (or internally-infected seed) among 10.000 seeds can be the source of one epidemic disease spreading in new crops (CHANG et al., 1991; DE LÉON et al., 2011). This ability of surviving for long periods in seeds, at least for 5 years (ASTA, 2015; EPPO, 2005), can be assigned to the fact that Cmm tolerates desiccation and cold temperatures (CHANG; RIES; PATAKY, 1991; WERNER et al., 2002).

The best way to avoid the disease on the field is to avoid the bacteria to reach new crops, by maintaining the seeds and transplants free of the pathogen, because nowadays there is no resistant cultivar nor effective shoot or root-applied bactericides and a few sanitary practices are important to disease control (JAHR et al., 1999; WERNER et al., 2002). Even if seed is routinely tested for Cmm and production of healthy transplants is an industry priority, sometimes small population densities can reach the field by infected seeds which often give rise to symptomless seedlings, which may carry latent infections or harbor the pathogen epiphytically. These asymptomatic seedlings may escape inspection

and cause epidemics after transplanting. Once this population enter into the field, will rapidly increase during the growing of the plant and outbreaks can remain unnoticed because symptomless seeds and seedlings and latent infections for long periods might occur (CARLTON; BRAUN; GLEASON, 1998; GITAITIS; BEAVER; VOLOUDAKIS, 1991; JAHR et al., 1999).

As new outbreaks and first reports of Cmm are still periodically noticed in different regions, successful control of tomato bacterial canker remains a serious problem for tomato cultivation worldwide. Probably, there are many other unpublished results on all continents and new outbreaks that can be masked in regions that were previously affected. The current losses due to this pathogen, although variable among years, may be very high at the global level (DE LÉON et al., 2011).

Provided that the use of pathogen free seeds is of prime importance for bacterial canker control, the main procedures to achieve this objective can be summarized in: (a) pathogen-free seed production fields; (b) seed sanitation; and (c) seed health testing. Phytosanitary measures should address these goals in a global mode in order to control tomato seed production with suitable procedures at all the process stages. Careful inspections of fields and analyses prior to their selection for seed production are required, mainly in developing countries in which available information about the presence and degree of spread of Cmm could be insufficient (DE LÉON et al., 2011).

Moreover, strict sanitary measures must be maintained in seed production fields to minimize the risk of pathogen introduction. Research on seed extraction and sanitation procedures is still needed because at the moment, a safe method that ensures the eradication of Cmm from naturally infected seeds without reducing seed germination is not available. Pathogen populations can be reduced greatly but not eradicated entirely (FATMI; SCHAAD; BOLKAN, 1991; PRADHANANG; COLLIER, 2009), making the detection of remaining

viable bacteria more difficult. Furthermore, seed treatments could also induce the appearance of injured or VBNC bacteria, preventing their growth on semi-selective media on which standard seed testing protocols for Cmm are based.

Therefore, the influence on pathogen viability of seed treatments currently used by the seed industry also requires further investigation. Standard protocols for detecting Cmm in tomato seeds are based on pathogen isolation and confirmation of pathogenicity (INTERNATIONAL SEED FEDERATION - ISHI, 2008), but true progress to improve pathogen isolation from seeds has been scarce in the last two decades (DE LÉON; SIVERIO; RODRÍGUEZ, 2006). Research on non-isolation methods, serological and mainly PCR-based techniques, has intensified in recent years (BACH et al., 2003; HADAS et al., 2005; KANESHIRO; MIZUMOTO; ALVAREZ, 2006; KOKOSKOVÁ; MRÁZ; FOUSEK, 2010; LUO et al., 2008; OZDEMIR, 2009; ZHAO et al., 2007), but in many cases they are only used as presumptive tests because of their inability to confirm the presence of viable and pathogenic cells required for a positive diagnosis (EPPO, 2013). However, there is a risk in using a commercial seed lot in which the pathogen has been detected by different techniques even though viable bacteria cannot be isolated. Accordingly, it seems necessary to improve and review the test schemes for diagnosis and detection of Cmm in seeds. The recent advances in Cmm genomics (GARTEMANN et al., 2003, 2008) and proteomics, together with microarray possibilities, could lead to the discovery of new targets for detection and diagnosis and, hopefully, innovative methods (LÓPEZ et al., 2008).

Among these recent advances in the last years, molecular genetic studies of Cmm helped to better elucidate the mechanism of pathogenicity. The genome of Cmm strain NCPPB382 is 3.298 Mb in size, with a high G+C content of 72.6% (GARTEMANN et al., 2008). Two large plasmids, pCM1 (27.5 Kb) and pCM2 (70 Kb), carry genes that are essential for virulence. Loss of either

plasmid delayed the progress of the wilting and a Cmm strain without any plasmids was unable to cause symptoms although it colonized tomato plants as an endophyte (MELETZUS et al., 1993). The first virulence gene identified in Cmm was *pat-1*, located on plasmid pCM2 (DREIER; MELETZUS; EICHENLAUB, 1997). The protein encoded by the *pat-1* ORF is a 29.7kDa serine protease. Several genes homologous to *pat-1*, which are involved in tomato-Cmm interactions, are found in pCM1 and the chromosome (BURGER et al., 2005; STORK et al., 2008). The *celA* gene, located on pCM1 and required for pathogenicity, encodes a 78Kda protein, CelA, which is similar to endo- β-glucanases of the cellulase family A1. Cel A may directly contribute to pathogenicity by degrading xylem vessel walls, allowing the spread of bacteria to adjacent tissue (JAHR et al., 2000). Several other extracellular enzymes produced by Cmm, including pectin methylesterase, polygalacturonase and xylanase, are also involved in cell wall degradation (BEIMEN et al., 1992; STRIDER, 1969). However, the genes encoding these enzymes and regulating their expression have not been identified (XU et al., 2010).

Furthermore, new data should be generated to provide a more complete picture of the life cycle of this tomato pathogen to help in developing more appropriate sampling and integrated methodologies for seed analysis.

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PAPER 1

Generation of tomato seeds artificially infected with a GFP – tagged strain of *Clavibacter michiganensis* subsp. *michiganensis*

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Abstract

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is an economically-important seed-borne pathogen that causes bacterial canker and bacterial wilt of tomato. In this study, a procedure was developed to generate tomato seed (internally-) infected with Cmm. These seeds can be used to evaluate methods for detection of Cmm in seed extracts, to study the effect of seed treatments and to study transmission from seed to seedlings. Seeds were subsequently wounded by scarification, softened by incubation on wetted blotting paper, and inoculated with a Cmm suspension by vacuum-infiltration. To enhance growth of the Cmm in the seeds, they were incubated on wetted blotting paper or on a Cmm-selective medium. Directly after inoculation, densities were 10^4 cfu seed^{-1} before or 10^3 cfu seed^{-1} if seeds were disinfected. Incubation of seeds resulted in a strong increase of Cmm populations to densities up to 10^{10} cfu seed^{-1} after 3 days. A GFP-tagged Cmm strain was used to visualize Cmm in plant material. The GFP signal could be observed by epifluorescence stereomicroscopy in germinated seeds on the seed coat, cotyledons, stem and roots. Colonization of xylem vessels in stems and

roots was observed with confocal laser scanning microscopy, indicating systemic infections of germinated seeds.

Keywords: bacterial canker, confocal laser scanning microscopy, epifluorescence stereomicroscopy, green fluorescent protein, seedlings

Introduction

Clavibacter michiganensis subsp. *michiganensis* (Smith) Davis et al. (1984) (Cmm), is a seed-borne pathogen causing bacterial canker and wilt in tomato (*Lycopersicum esculentum* Mill.), the most damaging bacterial disease of tomato (Eichenlaub and Gatermann, 2011). First discovered in Michigan (USA) greenhouses in 1909, it has now been reported to occur in most tomato production areas around the world (EPPO, 2005). Infected seed and transplants are responsible for long distance spread of the pathogen and the introduction into areas not previously occupied by Cmm (Chang et al., 1991; De Léon et al., 2011).

Seed may become infected unnoticed due to asymptomatic infections of tomato plants (Fatmi et al., 1991; Ricker and Riedel, 1993).

Infection of seeds can occur via colonization of the xylem vessels of peduncles, or after subsequent infection of fruit lesions, followed by translocation of the pathogen via the mesocarp and the vascular system of fruits tissues into the seeds (Tancos et al., 2013; Uematsu et al. 1977, Medina-Mora et al., 2001). Densities of Cmm in seed extracts are often low, but even low bacterial densities of 5-25 cfu per seed can result in transmission from seed to seedling (Kaneshiro and Alvarez, 2003; Van der Wolf et al., 2012). In addition, seed infection incidences as low as 0.01% can result in ca. 100 infection foci per hectare and in epidemics if conditions are conducive for secondary spread (Chang et al., 1991; Gitaitis et al., 1991, Gleason et al., 1993).

Secondary spread of Cmm on the field or greenhouses can occur via contaminated equipment used for clipping or grafting practices, worker's hand during the removal of lower leaves, disbudding or defoliation of seedlings and also touching symptomless leaves bearing Cmm-contaminated guttation droplets prior to touching nearby plants. Splashing water and strong wind during irrigation or soil-borne infections has also been described as important infection sources of secondary

spread (Kawaguchi et al., 2010; Ricker & Riedel, 1993; Sharabani et al., 2013).

Seed treatments to remove fruit debris such as seed fermentation and treatments with acids, and disinfection with warm water or with biocides will result in a decrease in the percentage of infected seeds, but it is assumed that internally-infected seeds will not be entirely free of bacteria. (De Leon et al., 2011, Dhanvantari and Brown, 1993, Strider, 1969; Grondeau and Samson, 1994).

During storage, the numbers of bacteria on the seeds may further drop but Cmm can tolerate desiccation and low temperatures relatively well (Chang et al., 1991; Werner et al., 2002) and can survive in tomato seeds for long periods (Bryan, 1930; Strider, 1969).

Seed certification programs play an important role in the management of bacterial canker, in which validated methods has to be used. For validation, also Cmm-infected seed lots are required, which are hard to find. Infected seeds can be generated via inoculation of peduncles prior to flowering of the tomato plants (Woudt, Syngenta, personal communication). However, this is an expensive and time-consuming

method and seeds harvested from these plants will be restricted to the seed testa.

The aim of our work is to develop and evaluate techniques to generate internally infected tomato seeds with Cmm. These seeds can be used for evaluation of detection methods, but also to evaluate the efficacy of seed treatments or to generate infected seedlings for studies on diseased tomato plants. Preferably internally-infected seed should be generated which are considered as a major threat as they will escape seed disinfections. To localize infections in seed and seedlings, we used a Cmm strain marked with green fluorescent protein, which allowed detection of the pathogen by fluorescence microscopy.

Materials and methods

Bacterial strains and growth conditions

Clavibacter michiganensis subsp. *michiganensis* strain IPO3525 was used, carrying the plasmid pK2-22-gfp (Chalupowicz et al. 2012), generated from Cmm strain IPO3356 (Bacterial Culture Collection of

Plant Research International). The spontaneous rifampicin resistant strain is highly virulent and stable expression of GFP *in planta* has been proven (Lelis et al., 2014). The strain was stored on beads (TS/70-YE, Protect bacterial preserves; www.tscswabs.co.uk) at -80°C and grown on tryptone broth with yeast extract (TBY) supplemented with kanamycin (Sigma) (50 μg ml^{-1}) and rifampicin (Sigma) (25 μg ml^{-1}) (per liter: tryptone 10 g, yeast extract 5 g, NaCl 5 g, pH 7.5 and agar 15 g) at 25°C. In tests with Cmm-eGFP in seeds, the medium was additionally supplemented with cycloheximide Sigma Chemical, St. Louis) to a final concentration of 200 μg ml^{-1} to prevent fungal growth. The parental strain Cmm IPO3356 was also used to compare growth rates and background fluorescence in the plating methods. In all experiments, Cmm was grown in TBY for 72 hours at 25°C prior to use. Cells were scraped from plates after adding 1 ml of a Ringer's solution to the plates (Oxoid) by using sterilized plastic loops. The optical density at 600 nm was set at 0.1 (approximately 10^8 cfu ml^{-1}) using a spectrophotometer (Ultrospec 10 Cell Density Meter - Amersham Biosciences).

Seed treatments

Different seed treatments or combination of seed treatments were used to generate seeds internally infected with Cmm. In all experiments, a single batch of tomato seed of cv. Nunhems TOP 1140 was used.

For vacuum – infiltration, the seeds were placed in a container with 100 ml of a bacterial suspension of 10^8 cfu ml^{-1} in sterilized Ringer's solution or in a Ringer's solution without bacteria as a negative control. The container was placed in a desiccator and submitted to a pressure of -600 mbar for 10 minutes and for 10 minutes more at atmospheric pressure. Half of the weight of the infiltrated seed lots were disinfected with 70% ethanol for 1 min and dried on sterilized blotting paper.

To facilitate the entry of the bacteria into the tomato seeds during inoculation, the seeds were shaken for 0, 1, 3, 5 and 10 minutes with sand and stones to create wounds. For this, the seeds were covered with sterilized silver sand and sterilized little stones or gravel (ratio 1:1:1), and shaken in 50 ml plastic flasks (Falcon Industries®) on a vortex at high speed. The sand and stones were removed with a sterilized sieve and tweezers.

As an alternative or to further facilitate bacterial entry, the seeds were incubated on blotting paper wetted with 8 ml of sterilized Ringer's solution (Oxoid) for 16 h at 4°C, 20°C or 25°C prior to inoculation, in order to soften seeds and to create entrances for the bacteria.

To enhance bacterial growth in seeds vacuum-infiltrated with Cmm, the seeds were incubated on blotting paper wetted with Ringer's solution or with nutrient broth. Ten tomato seeds were incubated on sterilized filter paper in 9 mm Petri dishes wetted with 8 ml of sterilized Ringer's solution (Oxoid) and incubated for 5 days maximum at 25°C. Similarly, they were placed on blotting paper wetted with 8 ml of undiluted, ten-times or hundred-times diluted TBY broth (Tryptone 10g, Yeast extract 5 g, NaCl 5 g, Distilled water 1000ml) supplemented with semi-selective antibiotics (Trimethoprim 80 mg l^{-1} and Nalidixic acid 20 mg l^{-1}) for 3 days maximum at 25°C. To prevent fungal growth, Nystatin 100 mg l^{-1}or Cycloheximide 200 mg l^{-1} was additionally added to the medium. Since seed germination on blotting paper with highly concentrated nutrient broth was delayed, the seeds were transferred after three days to other sterilized blotting paper wetted with 8 ml of sterilized

Ringer's solution (Oxoid) and incubated up to 5 days more at 25°C to enhance germination.

To determine densities of the Cmm population, individual seeds were crushed in 1 ml of Ringer's solution, and ten-fold serial dilutions were made until 10^{-7}. 100 µl of each dilution was pour-plated in 24 well plates in 300 µl of TBY supplemented with kanamycin (Sigma) (50 µg ml^{-1}) and rifampicin (Sigma) (25 µg ml^{-1}). Plates were incubated for 72 h at 25°C and fluorescent colonies were counted under an epifluorescence stereomicroscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter for the presence of a GFP signal under 495 nm blue light.

Microscopic studies

Seeds and seedlings, transferred into Petri dishes using sterilized forceps, were analyzed with ESM and by confocal laser scanning microscopy (CLSM) (Leica DM5500Q). Entire seeds were analyzed or after cutting the seeds longitudinally with a razor blade. The roots and stems of seedlings were separated with a razor blade. Plant material was

placed on a microscope slide with added sterile distilled water and a coverslip on top. Photographs were taken with a Leica Digital System (Leica) connected to the confocal laser scanning microscope.

Results

Vacuum-infiltration of untreated tomato seeds with a suspension of Cmm resulted only in an external contamination of seeds (figure 1). Cmm could not be detected on seeds disinfected with 70% ethanol even not after incubation on wetted blotting paper for five days. Conversely, Cmm populations on non-disinfected seeds increased during incubation from 10^3 to ca. 10^6 cfu seed^{-1}.

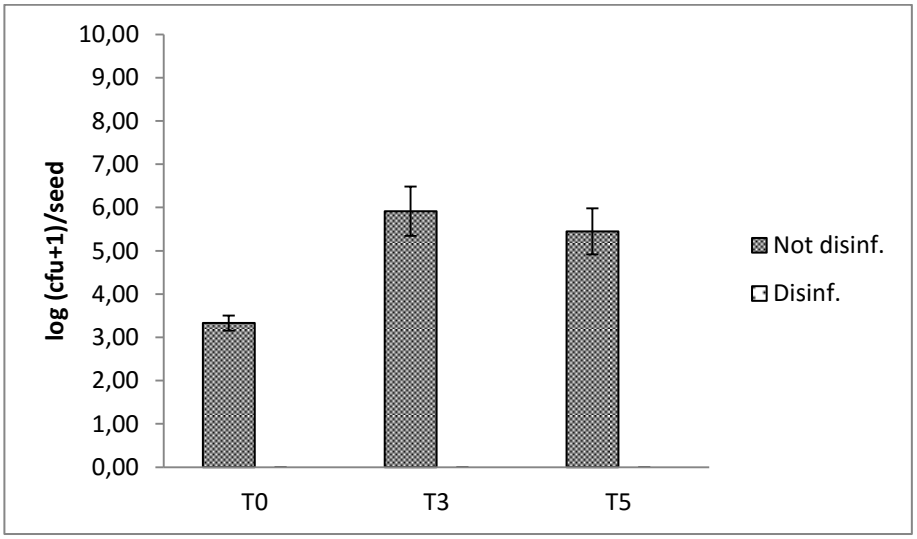


Figure 1. Population densities of a GFP-tagged *Clavibacter michiganensis* subsp. *michiganensis* strain in tomato seeds vacuum-infiltrated and incubated on wetted blotters for 0 (T0), 3 (T3) or 5 (T5) days at 25^{o}C. Seeds were incubated without disinfection and after disinfection with 70% ethanol. The densities of fluorescent bacteria were determined with pour plating (n=3). Bars are indicating the standard error.

To facilitate the entrance of Cmm during infiltration, the seeds were treated to create openings in the seed coat. The seeds were scarified by shaking with gravel and sand, resulting in a disruption of hairs from the integument (figure 2B). The scarified seeds were subsequently vacuum-infiltrated with Cmm and disinfected. After a scarification time of 5 or 10 minutes, viable Cmm cells were detectable in extracts of infiltrated seeds after seed disinfection. These populations were able to grow out to densities of 10^3 or 10^6 cells seed^{-1} during incubation on TBY supplemented with nalidixic acid and trimethoprim (TBY^{nt}) (figure 2A). Scarification, however, resulted in a rapid decline of the germination rate during storage (data not shown).

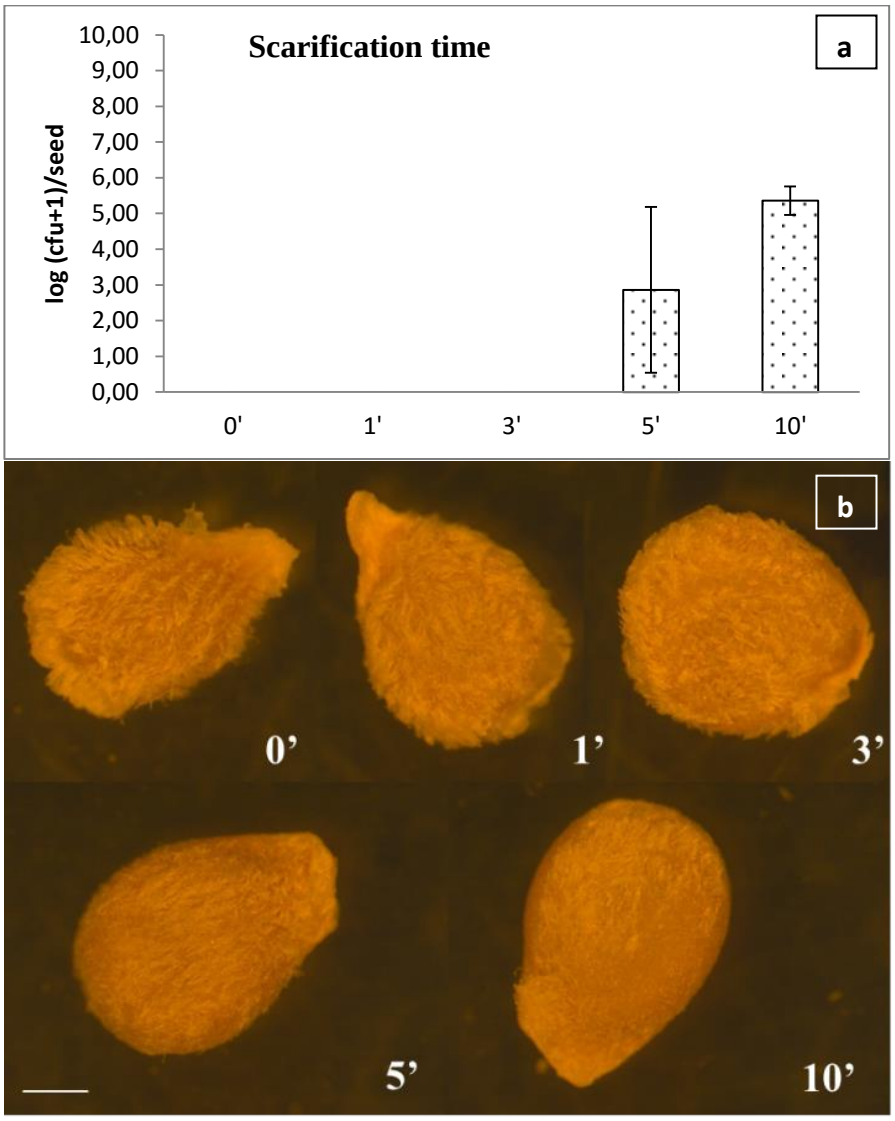


Figure 2. A. Population densities of a GFP-tagged *Clavibacter michiganensis* subsp. *michiganensis* strain in tomato seeds subsequently scarified for different time periods, vacuum-infiltrated, disinfected with ethanol and incubated on a blotter with a Cmm-selective media (TBY +

antibiotics) for 5 days at 25^{o}C. The densities of fluorescent bacteria were determined with pour plating (n=3). Bars are indicating the standard error. B. Photographs of tomato seeds scarified for different time periods (Scale bar – 6 mm).

To further support the entrance of Cmm, seeds were imbibed on wetted blotting paper for 16 h at 4, 20 or 25^{o}C after scarification but prior to vacuum-infiltration. For seeds imbibed without prior disinfection, initial densities were ca. 10^4 cfu seed^{-1}. Incubation on blotting paper wetted with a Ringer's solution resulted in a strong increase of Cmm populations in the first three days even up to densities of 10^{10} cfu seed^{-1}, but thereafter populations remained stable or even decreased (figure 3). In imbibed seeds that were disinfected after vacuum-infiltration, densities of Cmm were 10^3 cfu seed^{-1}. During incubation for five days, population densities only increased when the seeds were imbibed at 25^{o}C, but not at lower temperatures.

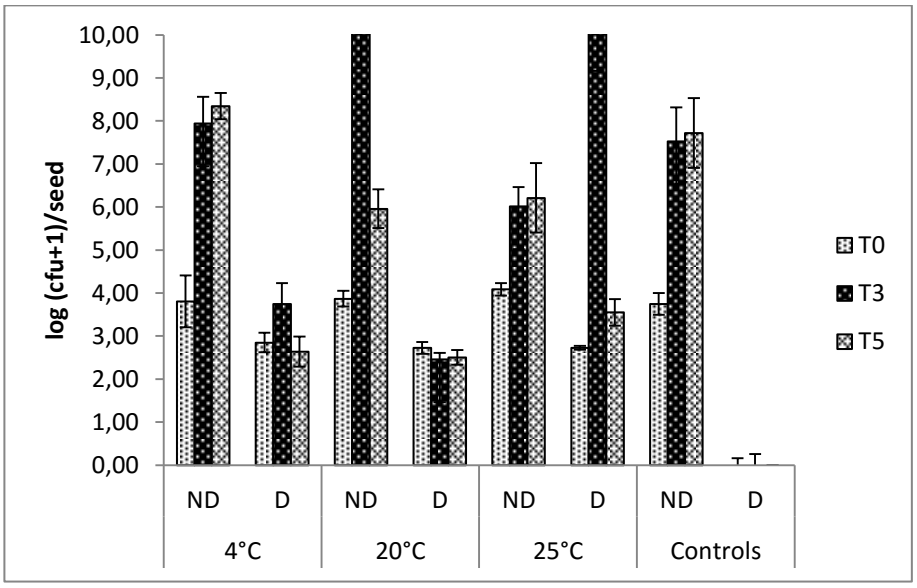


Figure 3. Population densities of a GFP-tagged *Clavibacter michiganensis* subsp. *michiganensis* strain in and on scarified seeds subsequently imbibed at different temperatures for 16h, vacuum-infiltrated with Cmm and incubated on blotters wetted with Ringer's for 0, 3 or 5 days at 25^{o}C. After vacuum infiltration, seeds were directly placed on blotters or first disinfected with 70% ethanol (ND – not disinfected seeds, D – disinfected seeds). Vacuum-infiltrated control seeds (C) were not scarified and not imbibed but directly inoculated via vacuum-infiltration and incubated on wetted blotters at 25^{o}C. For all treatments, 9 seeds were individually analysed. Bars are indicating the standard error.

To stimulate growth of Cmm in seeds, they were incubated on blotting paper drenched with a nutrient solution instead of a Ringer's solution. The growth was tested in undiluted, ten-fold or hundred-fold diluted TBY^{nt} or in TBY^{nt} supplemented with kanamycin and rifampicin, to further improve the selectivity of the medium for our target strain. Cmm in disinfected seeds incubated for a period of five days gradually grew from undetectable levels to population densities ranging between 10^5 and 10^7 cfu $seed^{-1}$ (figure 4). In non-disinfected seeds, Cmm populations grew in the first three days from 10^5 to between 10^8 and 10^{10} cfu $seed^{-1}$ after which densities remained stable or even decreased. Supplementing the TBY^{nt} with kanamycin and rifampicin did not further enhance growth of Cmm. No significant differences in densities were found for the three dilutions of TBY. However, seeds incubated on blotting paper with the undiluted TBY did not germinate, even after transferring non-germinating seeds to water drenched blotting paper (results not shown).

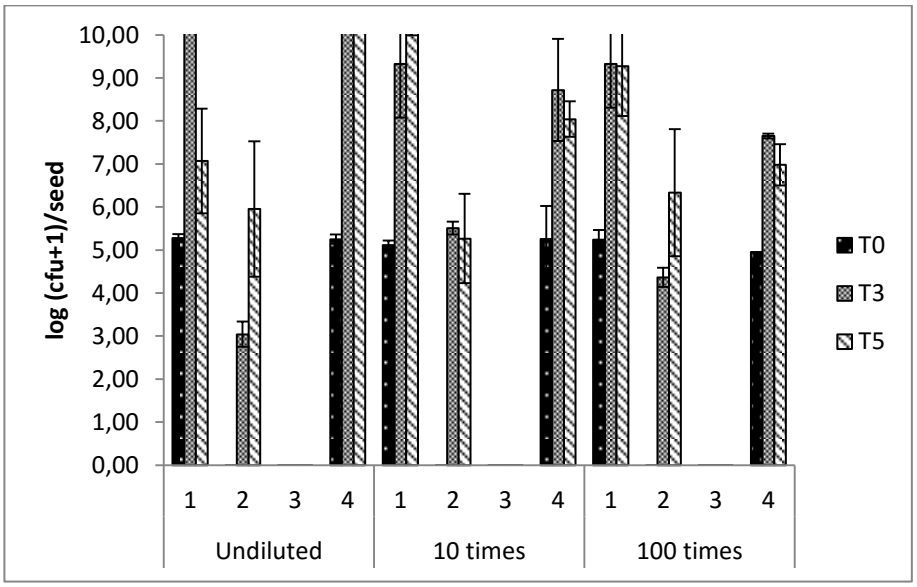


Figure 4. Population densities of a GFP-tagged *Clavibacter michiganensis* subsp. *michiganensis* strain in and on scarified seeds, vacuum-infiltrated with Cmm and incubated on blotters wetted with an undiluted, 10 times diluted or 100 times diluted semi-selective broth (TBY + Nalidixic acid and Trimethoprim). Seeds were analysed after 0, 3 or 5 days of incubation at 25°C. Treatment 1. Seeds vacuum-infiltrated but not disinfected. 2. Seeds vacuum-infiltrated and disinfected with 70% ethanol for 1 min. 3. Seeds not vacuum-infiltrated with Cmm (negative control). 4. Seeds vacuum infiltrated but not disinfected and incubated on a blotter with TBY supplemented with extra antibiotics (Kanamycin and

Rifampicin). The densities of fluorescent bacteria were determined with pour plating (n=9). Bars are indicating the standard error.

Population dynamics of Cmm in seeds incubated on blotting paper drenched in a Ringer's solution was also studied using epifluorescence stereomicroscopy (ESM) and for detailed studies with confocal laser scanning microscopy (CLSM). Directly after vacuum-infiltration, no fluorescent signal was detected with ESM on the seeds (data not shown). At 3 dpi (days post inoculation), clustered green fluorescent spots on external tissues of the stem were found on the non - disinfected, germinated seeds (figure 5a). A GFP-signal was also detected on the seed coat at the point of radicle protrusion and on root tissue (figure 5b). The fluorescent signal at the root tip could have been due to auto-fluorescence, as a similar signal was found in the control seeds (figure 5j). In disinfected seed, a GFP signal was found on roots, root hairs and seed coat, mainly at the point of radicle protrusion, and at the stem (figure 5c and 5d).

At 5 dpi, a GFP-signal was detected in 50-70% of the seedlings of disinfected and non-disinfected seeds. The signal was found on the seed

coat at the side of radicle protrusion (figure 5F and G), as well as localized GFP spots, more often on roots than on stems (figure 5H). In some of the seedlings from non-disinfected seeds, the GFP signal was further increased in seed tissues, in particular in the roots (figure 5E). The high homogeneous signal suggested a systemic infection of the tissues. A high GFP signal could also be found in rotten stems of seedlings (data not shown). No fluorescent signal was observed in water-treated (control) seedlings, except for autofluorescence on some of the root tips (figure 5I to L).

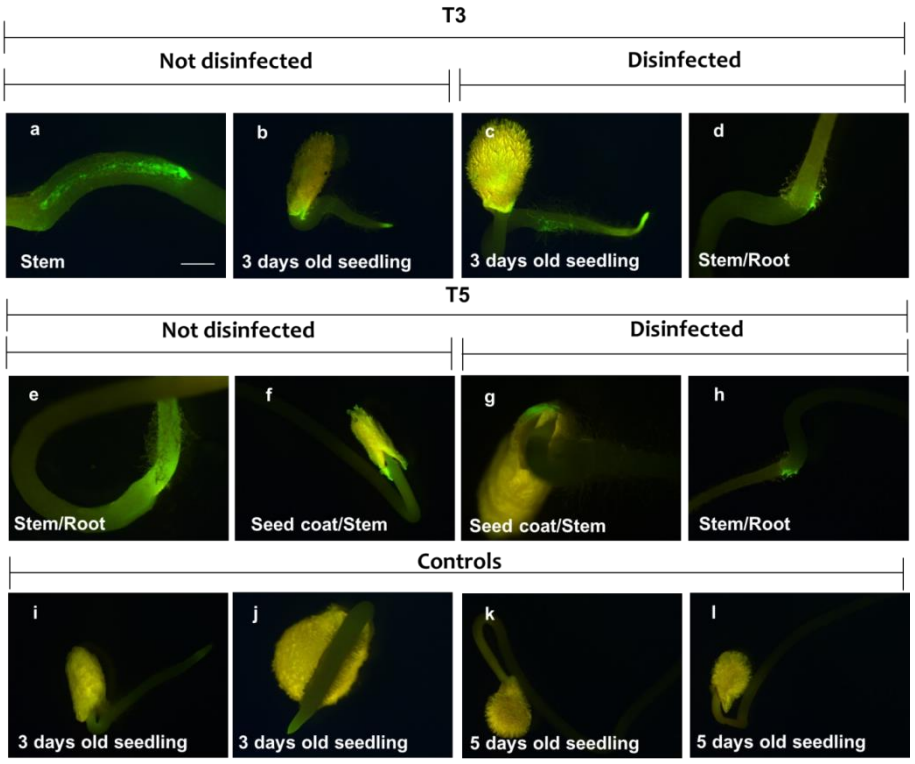
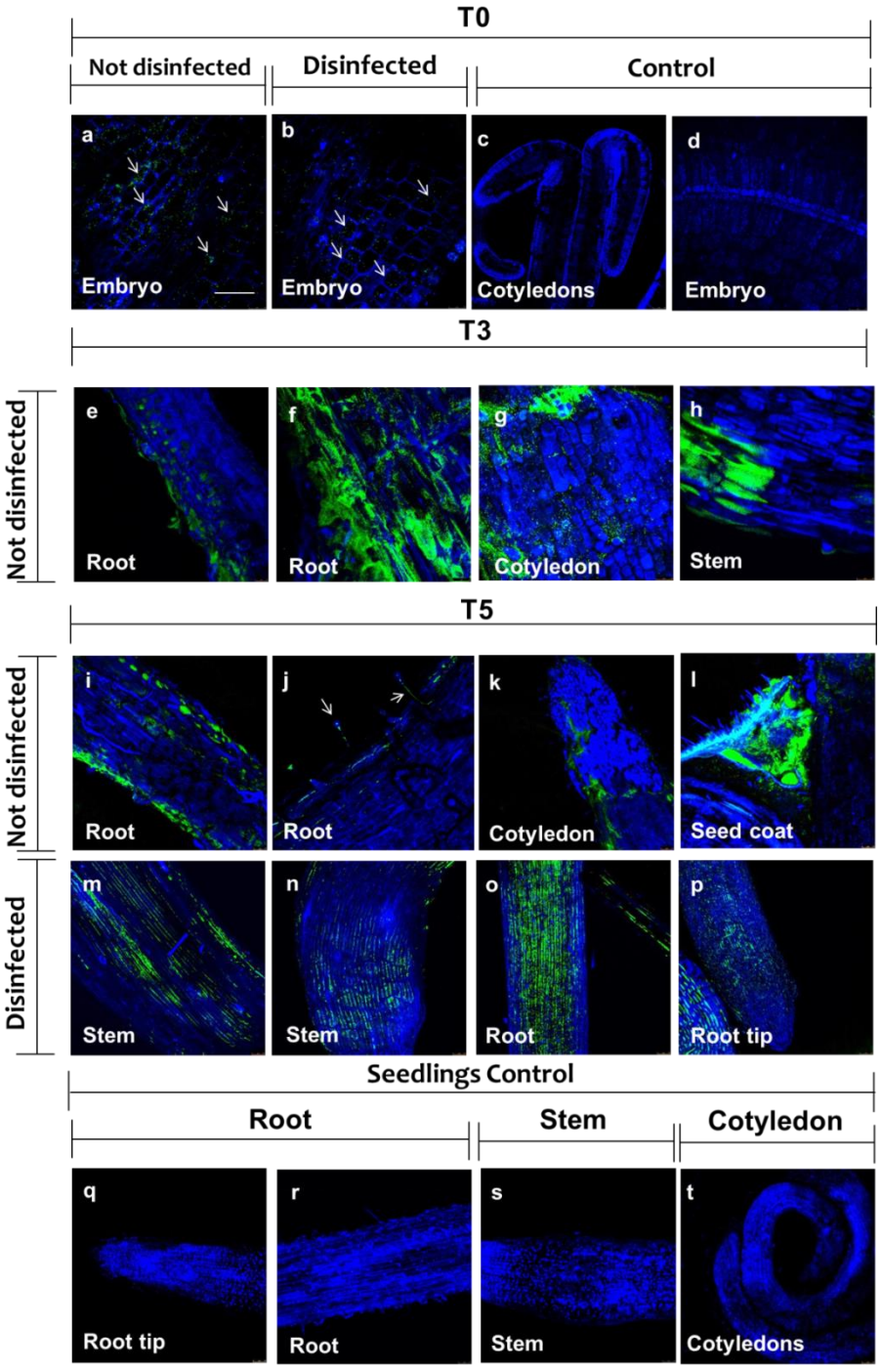


Figure 5. Epifluorescence stereomicroscope images of scarified tomato seeds vacuum-infiltrated with a GFP-tagged strain of *Clavibacter michiganensis* subsp. *michiganensis* or with water (control) and incubated on a blotter wetted with a Ringer's solution for 0 , 3 or 5 days at 25^{o}C. Vacuum-infiltrated seeds were disinfected with 70% ethanol or untreated before incubation on blotters. (Scale bar – 3 mm).

Analysis of seeds with confocal laser scanning microscopy (CLSM) a few hours after vacuum-infiltration with Cmm showed the presence of fluorescent cells in the intercellular spaces of the embryo (figure 6a and b), not present in water-inoculated seeds (figure 6c and d). At 3 dpi, Cmm populations increased strongly in seedlings grown from non- disinfected seeds. A high percentage (>80%) of analysed roots, cotyledons and stems of roots of seedlings were colonized with Cmm (figure 6e-h). High numbers of fluorescent cells were found in the intercellular spaces of the cortex and parenchyma of roots, stems and cotyledons of the embryo, frequently resulting in a disintegration of tissues. At 3 dpi, in seedlings grown from disinfected seeds, fluorescent cells were only found at a low incidence and in low densities (results not

shown). At 5 dpi, the seedlings were further developed. Fluorescent cells were observed in high densities in tissues of seedlings grown from non-disinfected and disinfected seeds. In the seedlings from non- disinfected seeds, fluorescent cells were observed again in stems and roots, more often externally than internally, and in cotyledons of the embryo (figure 6i, k, l). Occasionally, fluorescent cells were found in hair roots (figure 6j). Aggregated cells were also found under the seed coat at the point of radicle protrusion (figure 6l). In seedlings from disinfected seeds, the fluorescent cells were found more often in internal tissues of stems and roots, probably in xylem vessels as cells were found in vessels with a typical spiral structure (figure 6m, n, o). Cells were also found in the root tip (figure 6p). Fluorescent cells were not observed in any of the water treated control seedlings (figure 6q-t).


T0
Not disinfected
Disinfected
Control
a
Embryo
b
Embryo
c
Cotyledons
d
Embryo
T3
Not disinfected
e
Root
f
Root
g
Cotyledon
h
Stem
T5
Not disinfected
i
Root
j
Root
k
Cotyledon
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Root tip
Seedlings Control
Root
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Figure 6. Confocal laser scanning microscope images of transected tomato seeds and seedlings. Scarified seeds were vacuum-infiltrated with a GFP-tagged strain of *Clavibacter michiganensis* subsp. *michiganensis* or with water (control) and incubated on a blotters wetted with Ringer's for 0, 3 and 5 days at 25^{o}C. Vacuum-infiltrated seeds were disinfected with 70% ethanol or left untreated before incubation on blotters (Scale bar – 100 µm).

Discussion

Studies with tomato seed lots naturally-infected with Cmm are hampered by the lack of seed available. If available, they frequently contain low incidences of infected seeds of typically less than 0.1%, although higher incidences have been reported (Strider, 1969; Chang et al., 1991; Dhanvantari and Brown, 1993). Artificially-inoculated seeds have therefore been used to allow studies on detection, disinfection and transmission. Tsiantos (1987) used surface-sterilized, vacuum-infiltrated seeds, but infection incidences in seedlings grown from these seeds were low and unpredictable, varying between 0 and 25% between experiments.

Vacuum-infiltrated seeds were also used to evaluate seed treatments, but no information was provided on the localization of the inoculum (Kasselaki et al., 2011). Experiments with spray-inoculated seeds or seeds immersed in a bacterial suspensions, but also here information on the localization of Cmm in the seeds is lacking (Pradhanang and Collier, 2009; Milijasevic et al, 2009).

Herewith we describe an improved method to generate tomato seeds internally-infected with the seed-borne bacterial pathogen *Clavibacter michiganensis* subsp. *michiganensis*. These seeds may be used to improve and evaluate diagnostic methods including seed extraction procedures, for studies on seed treatments and on studies with infected plants. The method is based on creating entrances for the pathogen in the seed testa, followed by vacuum-infiltration of seeds and incubation of seeds under conditions favoring growth of Cmm in seed.

It is assumed that Cmm can be present internally in naturally-infected seed lots, although full evidence is lacking. During seed production, Cmm can be translocated via the vascular system or via floral parts into the seed, which makes internal infections likely (Medina-Mora et al., 2001; Tancos et al., 2013). It is further generally accepted that seed

treatments with biocides do not always result in full eradication of Cmm in tomato seed, probably due to the presence of internally-infected seeds that cannot be treated. The same is true for heat treatments of seed, in which the pathogen cannot be fully eradicated without considerable seed damage (Grondeau and Samson, 1994).

Vacuum-infiltration of untreated seed did not always result in the occurrence of internal infections. Seed hydration for only 20 min seems to be not long enough to make the testa permeable for infiltration of the bacteria. To enhance the infiltration of Cmm, several seed treatments were evaluated.

Scarification by shaking the seed with gravel and sand resulted in a visible disruption of hairs from the integument and consequently in a wounding of the testa, thereby giving entrance to the pathogen during infiltration. The wounding of the testa was also indicated by the decrease in the ability of scarified seeds to germinate. It is described that the testa plays an important role in the protection of seed against adverse environmental conditions (Debeaujon et al., 2000). The hairs are part of the thick epidermal layer of tomato seeds which during seed maturation partially breaks down, leaving the hairs behind (Getz et al., 1983). In our

studies, a positive relation was found between the time of scarification and the number of bacteria found in the scarified seeds after incubation on blotting paper.

The imbibition of seed prior to vacuum infiltration also resulted in the migration of Cmm into the seed. During imbibition, cracks in the seed coat, which may be a portal for Cmm to enter the seeds, are filled with water. In soy bean, cracks of up to 5 µm were found, wide enough for the bacterium to enter (Ma et al., 2004). Cotyledon swelling during water migration may result in a further distortion of the testa (Koizumi et al., 2008), and may further enhance the migration of bacterial pathogens into the seed.

Only relatively low numbers of bacteria are able to enter the seed coat, even after enhancing penetration of seed via scarification or imbibition. In some experiments, Cmm was only detectable after incubation of the seeds on blotting paper for at least three days (figure 4). In other experiments, up to 1000 cfu $seed^{-1}$ could be detected in seed directly after disinfection of seed (figure 3). In seed naturally-infected with Cmm or after inoculation of the peduncle, similar small densities have been found per seed (Kaneshiro and Alvarez, 2003; Van der Wolf et

al., 2012). These low densities of bacteria of 5–25 cfu per seed, however, are already sufficient for studies on transmission of Cmm from seed to seedling, whereas densities higher than 600 cfu per seed can result in non-germinability of seed (A.M. Alvarez, personal communication, University of Hawaii at Manoa). Highly infected seed may therefore have a limited significance in the epidemiology but they are required to visualize Cmm in seed with microscopic techniques.

To enhance multiplication of Cmm in seeds after vacuum-infiltration, they were incubated on blotting paper wetted with Ringer's, a salt solution, or wetted with (diluted) TBY with antibiotics (TBY^+), a selective nutrient broth. On blotting paper with Ringers, Cmm population densities hardly increased, but with TBY^+ populations in disinfected seed increased to approximately 10^6 cfu $seed^{-1}$. This indicates that diffusion of the nutrients and selective antibiotics from the blotting paper into the seed allowed specific growth of Cmm populations to grow. In the same period, background population densities hardly increased (data not shown).

Seeds incubated on blotting paper with Ringer's or 100-times diluted TBY^+ germinated well, but on undiluted TBY^+ seeds did not germinate, probably due to a high salt concentration resulting in an

increased osmotic pressure (Demir and Mavi, 2008; Falleiro et al., 2010; Machado et al., 2008; Zhang et al., 2010). Germination may also be inhibited by the presence of the antifungal compound cycloheximide in the TBY^{+}. Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms including plants, by interfering with the translocation step in protein thus blocking translational elongation (Schneider-Putsch et al., 2010). Germination was not affected if cycloheximide was substituted by Nystatin, a polyene compound, specifically inhibiting moulds and fungi (data not shown).

In our studies, Cmm did not affect seed germination and during the maximum incubation time of five days no visible symptoms in seedlings were observed, possibly because population densities were too low. Cmm has to establish a population density of at least of 10^{8} cfu g^{-1} of plant tissue in order to induce disease symptoms (Gartemann et al., 2003) and after inoculation of plant material, more than five days are required for disease development (Balaji et al., 2008; Lelis et al., 2014).

To visualize Cmm in plant material two markers have been used so far, i.e. bioluminescence (Xu et al., 2010; Xu et al., 2011) and fluorescence (Chalupowicz et al., 2012; Lelis et al., 2014; Tancos et al.,

2013). Bioluminescence allows the non-destructive monitoring of the internal colonization of bacteria in relatively large plant parts. It has been successfully used to study the distribution of Cmm in young tomato plants and to assess host susceptibility (Fukui et al., 1996; Xu et al., 2010; Xu et al., 2011). However, this methodology cannot monitor single cells and can only detect metabolically-active cells as the technique depends on an active cellular uptake of substrates (Kohlmeier et al., 2007). The use of green fluorescent protein as a reporter largely circumvents these problems. Several Cmm strains have been transformed with an eGFP gene set for stable expression of the protein (Chalupowicz et al., 2012; Lelis et al., 2014).

Confocal laser scanning microscopy indicated that subsequent scarification, vacuum-infiltration with Cmm and incubation on blotting paper resulted in infections of testa and embryotic tissues. A GFP-signal was found in embryonic tissues of non-disinfected and disinfected tomato seeds, which were not found in water-inoculated control seeds. Interestingly, Cmm seems to colonize embryonic tissues both inter- and intracellularly, although artefacts due to smearing during dissection of seeds cannot be fully excluded. The localized intracellular colonization of

parenchymatic tissues in stems and roots of seedlings, however, is undisputable and cannot be a result of artefacts. Cmm cells have also been found to colonize mesocarp cells of tomato fruits intracellularly (Tancos et al., 2013). Most phytopathogenic bacteria remain in the intercellular spaces or apoplast, although some have been reported to invade the intracellular spaces (Hogenhout and Loria, 2008; Joshi et al., 2007; Sharma et al., 2001). For intracellular colonization, the barrier of the primary or secondary cell wall consisting of cellulose fibers has to be overcome. For this, Cmm produces as one of the major virulence factors endo-β-1,4-glucanases that cleave β-1,4-glycosidic bonds of these fibers internally (Jahr et al., 1999).

In five-old day seedlings, a GFP-signal was found in stems and roots, which may have occurred from embryonic infections but also from contact with the infected testa during radicle protrusion. In the roots, a fluorescent signal was found in the epidermis, parenchyma cells of the cortex and in vascular tissue in the stele. Incidentally root hairs were found to be colonized, showing that they may act as port of entry for Cmm, as has been reported for other pathogens of tomato plants (Bryan, 1930; Getz et al., 1983). In the stems, a GFP-signal was found in the

epidermis, parenchymatic cortex tissue and in the pith. If these densely populated, internally-infected seedlings are used, disease expression is to be expected.

In conclusion, an efficient method was developed to generate tomato seed infected with Cmm, which can be used for the validation of detection methods for Cmm, studies on the transmission from seed to seedlings and for studies on the efficiency of seed treatments.

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(VERSÃO PRELIMINAR)

PAPER 2

Population dynamics, distribution and incubation of *Clavibacter michiganensis subsp. michiganensis* in vacuum-infiltrated tomato seed prior to detection

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Abstract

Bacterial canker and bacterial wilt, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), is one of the most important seed-borne diseases of tomato. For studies on colonization of tomato seeds, seed treatments, seed transmission and for evaluation of diagnostic methods with Cmm, there is a need for seed that is internally infected with Cmm, how it is observed in natural infections. Seeds were scarified and vacuum-infiltrated with a GFP-tagged and spontaneous rifampicin resistant strain that was incubated on a semi-selective medium to increase the Cmm population. The population dynamics of Cmm on or in (disinfected) seeds were studied by plating, a non-invasive monitoring system for GFP-tagged bacteria in plants (PathoScreen), Confocal laser scanning microscopy (CLSM), Epifluorescence stereomicroscopy (ESM) and a TaqMan assay. Also, dead and viable seeds were used to observe the Cmm behavior on and in different seed tissues. The population densities of Cmm rapidly increased from 10^3 cfu per seed, 3 hours after incubation, to 10^{10} cfu per seed after 72 hours on both viable and dead seeds. Internal

infections was observed in viable seeds from non-detectable Cmm cells right after the inoculation to ca. 10^7 cfu per seed after 5 days of incubation on selective medium. The increase of the bacteria into the seed was confirmed with the monitoring of the GFP signal by the PathoScreen analyzes. Transected and undivided seeds analyzed with CSLM and ESM indicated that during incubation, Cmm colonized trichome hairs, the outer testa layers, the endosperm and embryo. Previous incubation of infected seeds in semi-selective media improved the detection of Cmm by a TaqMan assay. This enhancement was obtained with the increasing of low population densities of Cmm in tomato seeds.

Keywords: bacterial canker, confocal laser scanning microscopy, epifluorescence stereomicroscopy, green fluorescent protein, TaqMan assay, qPCR.

Introduction

Bacterial canker of tomato (*Solanum lycopersicum*, syn. *Lycopersicon esculentum* Mill.) is caused by *Clavibacter michiganensis*

subsp. *michiganensis* (Smith) Davis et al. (1984) (Cmm), a Gram-positive bacterium which is considered to be the most serious seed-borne pathogen of tomato. Cmm is responsible for major economic losses and is found in most production areas of tomato around the world. Cmm is a quarantine bacterium in many countries, including the EPPO region (Eichenlaub et al. 2006).

Infected seeds are responsible for long distance spread (Tsiantos, 1987; Strider, 1969) and for new outbreaks in areas previously regarded free of bacterial canker (Gitaitis et al. 1991; Fatmi et al. 1991; Tsiantos1987).

The pathogen can survive for many years on seed and, in particular, internal seed infections are difficult to eliminate via seed treatments, even when combined with antibiotics (Strider, 1969; Tsiantos, 1987).

Methods for seed extraction and chemical or thermal seed treatments have been investigated to provide an effective procedure to obtain seeds free of this pathogen. Some methods are effective for reducing Cmm populations, but there is no available method that ensures the complete eradication of this pathogen from naturally infected seeds

without decreasing seed germination (Pradhanang and Collier, 2009). Therefore, the influence of seed treatments currently used by the industry on the viability of the pathogen also requires further investigation, because with the chemical treatments, the pathogen could became quiescent or enter into the viable but nonculturable state (VBNC), making efforts for his detection on seeds even more complicated (De Leon et al., 2011; Pradhanang and Collier, 2009).

The production of Cmm-free tomato seeds is the most important strategy to control of the disease and is based on preventing the introduction of the pathogen in the field, since the seed treatment have not been sufficient or affect seed germination, especially if the seeds are internally infected (Fatmi et al., 1991; Dhanvantari, 1989).

To detect this harmful pathogen, fast and accurate methods are required, able to detect the pathogen in seeds, both superficially and internally, also in case of low infection incidences which often result in low densities in seeds (León et al., 2011).

To develop and evaluate methods for detection, as well as to study seed transmission and the efficacy of seed treatments, Cmm-infected seeds are required. The availability of naturally-infected seeds is limited

and often these seed lots only have low infection incidences (Dhanvantari and Brown, 1993). As an alternative, infected seeds can be produced by inoculation of peduncles of tomato plants, which result in a translocation of Cmm to the developing seeds (Woudt, Syngenta, pers. comm.). However, these infections are mainly located in or on the seed coat (Woudt, Syngenta, pers. comm.) and, preferably, internal infections of tomato seed are required to simulate the natural conditions in the transport of Cmm to the field.

The aims of our study were measure and observe the dynamics and distribution of Cmm population on and in tomato seeds, using both dead and viable seeds. A method to improve detection of Cmm in seeds using a TaqMan assay was also tested. For this, tomato seeds were vacuum-infiltrated with Cmm and incubated under various conditions to allow development of Cmm in seeds. To visualize the pathogen in the seed and to enhance the enumeration with dilution plating methods, a GFP-tagged spontaneous rifampicin resistant strain of Cmm was used. The pathogen was monitored in seeds using PathoScreen, a non-invasive system allowing sensitive monitoring of fluorescent signals in plant

material, Confocal laser scanning microscopy (CLSM) and Epifluorescence stereomicroscopy (ESM).

Material and Methods

Bacterial strains and growth conditions

Clavibacter michiganensis subsp. *michiganensis* IPO3356, highly virulent and rifampicin resistant (Rif^+), was grown on tryptone broth with yeast extract (TBY) (per liter: tryptone 10 g, yeast extract 5 g, NaCl 5 g, pH 7.5 and agar 15 g) at 25°C for 3 days. TBY medium was supplemented with rifampicin to a final concentration of 25 μg mL^{-1} and cycloheximide ($Cyclo^+$) (Sigma Chemical, St. Louis) to a final concentration of 200 μg mL^{-1} to prevent fungal growth. Cmm GFP-tagged IPO3525 (Cmm strain IPO 3356 transformed with plasmid pK2-22-*gfp*) (Lelis et al., 2014) was grown in Luria Bertani (LB) agar media (per liter: tryptone 10 g, yeast extract 5 g, NaCl 10 g), supplemented with kanamycin (Kan^+) (Sigma) to a final concentration of 50 μg mL^{-1},

rifampicin (25 μg mL^{-1}) and cycloheximide (200 μg mL^{-1}) (Sigma Chemical, St. Louis) at 28°C for 3 days.

Seed sterilization and freezing method

To prevent germination in order to facilitate microscopic observations of the pathogen in seeds during incubation on the semi-selective media (TBY^{+} – TBY + 25μg mL^{-1}Rif + 50μg mL^{-1}Kan + 200μg mL^{-1}Cyclo), seeds were killed by freezing and thawed back, using the protocol described by Limonard (1966) with modifications. Tomato seeds (*Lycopersicon esculentum* Mill.) cultivar 'Moneymaker', were surface sterilized by immersions in 1% NaOCl (Sigma) for 20 minutes and washed four times in sterile distilled water. Surface-sterilized tomato seeds were placed on a moist blotter (wetted with Ringer's solution – Oxoid) for 24 hours at 25°C and then put into freezer at -20°C for 24 hours. Killed seeds were inoculated by vacuum infiltration as described below, placed in 9 mm Petri dishes containing the respective agar medium for each type of inoculum and incubated at 25°C in the dark.

Seed inoculation

Viable or dead tomato seeds (cv. Moneymaker) were inoculated by vacuum infiltration. Approximately 1000 seeds (3 grams) were submerged in a beaker with 100 mL of a suspension of the GFP-tagged Cmm strain IPO3525 or the parental strain IPO3356 with a density of ca. 10^8 cfu mL^{-1}, or alternatively in distilled sterile water as a negative control. They were placed for 10 min in a desiccator at a vacuum of -600 mBar, followed by 10 min incubation at atmospheric pressure. The suspension was removed and the seeds were dried back in sterilized blotter papers. Two hundred viable or dead inoculated seeds were placed in 9 mm Petri dishes containing an agar medium. Seeds inoculated with water (negative control) were placed on TBY medium supplemented with cycloheximide to a final concentration of 200 μg mL^{-1}. Seeds inoculated with the wild type strain (IPO3356) were placed on TBY medium supplemented with rifampicin to a final concentration of 25 μg mL^{-1} and cycloheximide (200 μg mL^{-1}). Seeds inoculated with the GFP strain (IPO3525) were placed on TBY medium supplemented with rifampicin to

a final concentration of 25 μg mL^{-1}, kanamycin to a final concentration of 50 μg mL^{-1} and cycloheximide (200 μg mL^{-1}).

Stability of Cmm GFP-tagged in seeds

At 3, 72 and 120 hours post inoculation (hpi), seeds were transferred to sterilized 9mm Petri dishes, sterilized by spraying with 70% ethanol and washed in sterile distilled water. Individual seeds were macerated with sterile distilled water (1000 μl $seed^{-1}$), and 100 μl of eight ten-fold serial diluted macerates were plated onto LB medium plus kanamycin (GFP-tagged strain), or onto LB medium plus rifampicin (parental strain), or onto LB medium (water treated control) (n=3). The ratio of GFP-positive and GFP-negative bacterial colonies on plates was determined at four days after incubation at 25°C.

PathoScreen analyzes

PathoScreen, developed at Wageningen UR and manufactured by PhenoVation B.V., produces 12 megapixel images representing the

intensity of GFP fluorescence in 16-bit data format (Van der Lee et al., 2009). PathoScreen is a non-invasive monitoring system in which lasers are combined with a sensitive, high-resolution, cooled camera to monitor bacteria labelled with a fluorescent protein in intact plant material. Fluorescence signals are recorded with a sensitive charge-coupled device (CCD) digital camera and stored for digital image analysis. After correction for autofluorescence, the surface area of affected tissue is assessed by determining the number of GFP-positive pixels in images of the surfaces of the plant material, and hence an estimation of the proportion of affected tissue. It was used to analyze dead and viable tomato seeds infected with the GFP-tagged Cmm strain (IPO 3525), the parental strain (IPO 3356) or water.

Seeds were analyzed 3, 72 and 120 hpi (experiment with undivided dead seeds) and 0, 3 and 5 days after inoculation (experiment with transected viable seeds). In both experiments, seeds were incubated in TBY^{+} at 25°C. In the test using viable seeds, half of the seeds were disinfected with ethanol 70% for 1 minute after the inoculation to check only internal infections. Fluorescence and autofluorescence were induced by laser radiation. Three intact or transected seeds were used for each

strain of Cmm (and the negative control) per hours or days post inoculation (hpi/dpi). This experiment was done once for dead seeds and twice for viable seeds.

Confocal laser scanning microscopy and Epifluorescence Microscopy

Dead and viable seeds inoculated with the GFP-tagged Cmm strain (IPO 3525), the parental strain (IPO 3356) or water were collected from the medium at 3, 72 and 120 hours after inoculation and washed in sterile water. In the test using viable seeds, half of the seeds were disinfected with ethanol 70% for 1 minute after the inoculation to check also the Cmm population grown only from internal infections. Transverse and longitudinal cuts were made with a sterile razor blade. These cuts were placed on a microscope slide with distilled water and observed under a Confocal laser scanning microscope (CLSM) (Leica DM5500Q). Photographs were taken with a Leica Digital System (Leica) connected to a CLSM. For the Epifluorescence stereomicroscopy (ESM) studies, seeds directly after inoculation, 3 and 5 days after inoculation were put into empty Petri dishes with sterilized tweezers, transverse cuts were made

with a sterile razor blade, and examined for the presence of a GFP signal under 495 nm blue light using an epifluorescence stereo microscope (Leica Wild M32 FL4) equipped with a mercury high pressure photo-optic lamp (Leica Hg 50W/AC) and GFP plus filter. For the microscopy studies, seeds were incubated in TBY^{+} at 25°C.

Incubation of vacuum-infiltrated seeds prior to detection

To enhance the capacity of Cmm detection in the seeds, the Cmm population was increased prior to detection with a TaqMan assay. Seeds were shaken (scarified) for 10 minutes with sand and stones to create wounds and facilitate the entry of the bacteria into the tomato seeds during inoculation. For this, seeds were covered with sterilized silver sand and sterilized little stones or gravel (ratio 1:1:1), and shaken in 50 mL plastic flasks (Falcon Industries®) on a vortex at high speed. After that, seeds were vacuum – infiltrated with a Cmm suspension (as described above), half of the seeds were disinfected with ethanol 70% for 1 minute and dried back on sterilized blotters (to analyze only internal infections)

and not disinfected, disinfected and negative control samples were incubated in TBY^+ for 0, 3 and 5 days at 25°C.

Another method was tested to increase Cmm population in tomato seeds, as an alternative for a cheaper technique. Bacterial entry into the seeds was facilitated by scarification (as described above) and incubation on blotting paper wetted with 8 mL of sterilized Ringer's solution (Oxoid) for 16h at 25°C prior to inoculation, in order to soften the seed coat. Next, seeds were vacuum-infiltrated with Cmm solution and incubated on blotting paper wetted with ten times (1/10) diluted TBY^+ broth (Tryptone 10g, Yeast extract 5 g, NaCl 5 g, Distilled water 1000mL), or with Ringer's solution (negative control) for 2 days. After that, the seeds were transferred to wetted blotters and incubated for 0, 3 and 5 days at 25°C. In every time day point, densities of the Cmm population were determined by crushing individual seeds in 1 mL of Ringer's solution, and ten-fold serial dilutions were made until 10^{-7}. 100 µl of each dilution was pour-plated in 24 well plates in 300 µl of TBY^+. Plates were incubated for 72h at 25°C and fluorescent colonies were counted under an epifluorescence stereomicroscope (ESM).

TaqMan Assay

After incubation in semi-selective media as described before, seed extracts were analyzed with a TaqMan assay to confirm the presence of Cmm. A water sample was included as a negative control. A Cmm seed extract supplemented with a ten-fold serial dilution of Cmm ranging from 10^8 to 10^0 cells seed^{-1} was included as a reference control. DNA extraction was performed with a Kingfisher TM robot (Thermo Fisher Scientific Oy, Vantaa, Finland.) with the sbeadex® mini plant kit according to the manufacturer's instructions (LGC Genomics, Beverly, USA). The TaqMan assay was conducted using the RZ_Ptssk primers (Forward primer RZ_Ptssk10: 5'-GGG GCC GAA GGT GCT GGT G-3'; Reverse primer RZ_Ptssk11: 5'-CGT CGC CCG CCC GCT G-3'; RZ_Ptssk12: Probe 5'-Fam-TGG TCG TCC /ZEN/TCG GCG CC/IABkFQ -3') (Sen et al., 2013). The reaction mixture included 12.5 µl Takara mix, 0.4 µl Rox II, 0.75 µl of each F/R primer (10 µM), 0.5 µl of probe (5µM), 8.1 µl of Milli-Q water and 2 µl of DNA with a final volume of 25 µl. Reactions were performed on an ABI 7500 Real Time PCR System (Applied Biosystem). The thermo cycling conditions were 2

minutes at 95°C, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Results were considered positive if the Ct values were lower than 35.

Results

In viable seeds, the population dynamics of both Cmm strains, IPO3525 and IPO3356, followed the same pattern, gradually increased from ca. 10^3 cfu $seed^{-1}$ to ca. 10^{11} cfu $seed^{-1}$ at 120 hpi (Fig. 1B). However, in dead seeds, the populations of Cmm IPO3525 gradually increased from ca. 10^3 cfu $seed^{-1}$ to ca. 10^{11} cfu $seed^{-1}$ at 120 hpi and the populations of Cmm IPO3356, initially increased in densities up to ca. 10^{11} cfu mL^{-1} after 72 hpi but then decreased to a density of ca. 10^3cfu $seed^{-1}$ at 120 hpi (Fig. 1A).

We presumed that Cmm population in this experiment was mainly present on the seed coat and seed trichomes and could not penetrate the deepest tissues of the seed. That's because in our first tests with internal infections, we found that after inoculation of tomato seeds with a Cmm suspension and surface disinfection, Cmm cells were not found in seed

samples and was not observed the development of the disease after sowing these seeds in a greenhouse (data not shown).

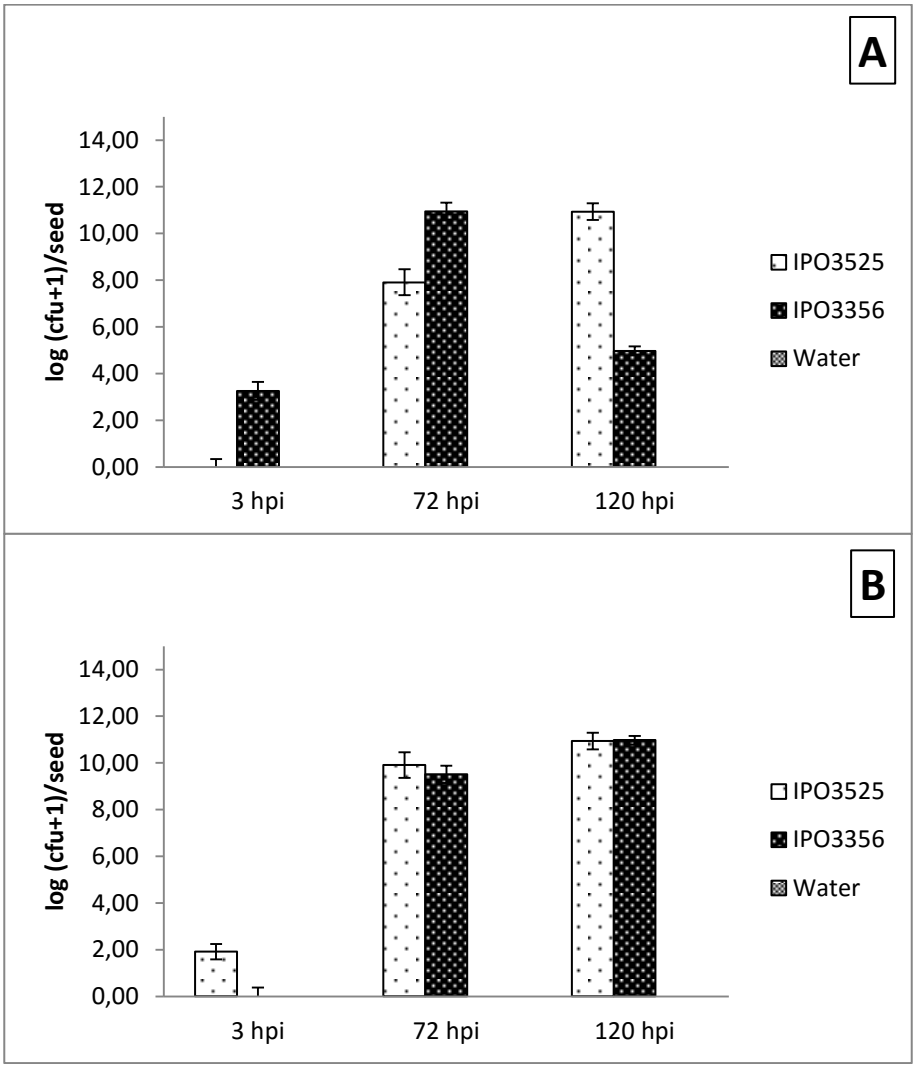

Figure 1. Population dynamics of the GFP-tagged *Clavibacter michiganensis* subsp. *michiganensis* strains IPO 3525 and the wild type strain IPO 3356 in dead (A) and viable (B) tomato seeds. The average

densities in seeds were determined at 3, 72 and 120 hpi (n=3). The vertical bars indicate the standard errors.

For that reason, we suspected that other ways to get Cmm cells inside of the tomato seed were needed. In previous studies, we found that scarification of the outer testa layers facilitates the bacterial entrance into the seeds during the vacuum-infiltration. After the scarification and inoculation of tomato seeds with a Cmm suspension, half of the seeds were superficially disinfected to remove those Cmm cells from the seed coat and let only those who got inside of the seed to grow during incubation. The incubation of these seeds on a semi-selective media improved the densities of Cmm from ca. 10^5 cfu seed^{-1} to higher than ca. 10^{10} cfu seed^{-1} on/in not disinfected seeds (Fig. 2A). In the disinfected seeds, Cmm population grown from undetectable cells right after the inoculation, to ca. 10^7 cfu seed^{-1} after 5 days of incubation (Fig. 2A), showing that from a few single cells, a high density of Cmm population is achieved with the right type of incubation and grow stimulation.

Increasing bacterial population on/in seeds is a required method to detect those bacterias that commonly occur in a low incidence in seeds.

Certified laboratories for detection of phytobacteria in seed samples are always searching for lower cost techniques but that doesn't lose in efficacy. As a cheaper method, the incubation of tomato seeds vacuum-inoculated with a Cmm suspension on a diluted semi-selective broth increased the Cmm population to an approximately ca. 10^7 cfu seed^{-1} in not disinfected seeds after 2 days of incubation (Fig. 2B). Further incubation time on wetted blotters to stimulate the bacteria growth did not enhanced the Cmm population, but the population levels remained the same (Fig. 2B). Still, the internal population in superficially disinfected seeds increased again from undetectable Cmm cells, even after 2 days of incubation on a nutrient broth, to a ca. 10^6 cfu seed^{-1} after 5 days of incubation on wetted blotters (Fig. 2B).

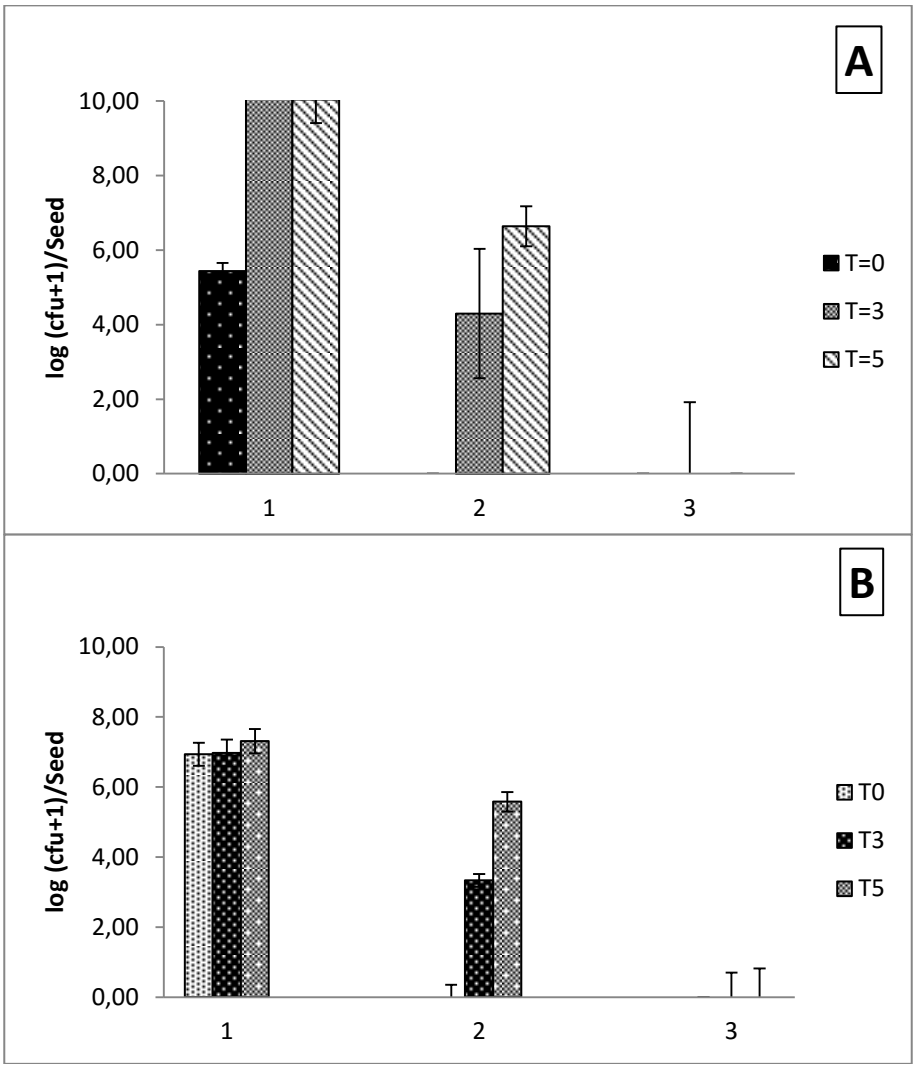

Figure 2. Population densities of a GFP-tagged *Clavibacter michiganensis* subsp. *michiganensis* strain in and on scarified seeds, vacuum-infiltrated with Cmm and incubated on Cmm semi-selective medium (TBY+ antibiotics) (A) and seeds vacuum-infiltrated with GFP-Cmm, incubated for 16 hours on wetted blotters at 25°C and 2 days incubated on blotters

wetted with an 10 times diluted semi-selective broth (TBY + antibiotics) (B). Seeds were analyzed after 0, 3 or 5 days of incubation at 25°C. Treatment 1. Seeds vacuum-infiltrated but not disinfected. 2. Seeds vacuum-infiltrated and disinfected with 70% ethanol for 1 min. 3. Seeds not vacuum-infiltrated with Cmm (negative control).The densities of fluorescent bacteria were determined with pour plating (n=9). Bars are indicating the standard error.

After 5 days post inoculation (120 hpi), all colonies re-isolated from both dead and viable seeds, which had been inoculated with Cmm strain IPO3525, showed a high expression of GFP as observed when compared with the parental strain IPO3356 (Fig. 3C). With Epifluorescence stereomicroscopy, a high GFP-signal was also observed on the seed coat from viable not disinfected seeds after 3 days of incubation on TBY^{+}, which increased at 5 dpi (Fig. 3B, Fig. 4, Fig. 5B). However, on disinfected seeds, the seed coat showed just a few spots of fluorescent signal, even after 5 days of incubation, demonstrating that even if high levels of Cmm population can be found infecting internal tissues of tomato seeds, the fluorescent signal remains under the seed coat

(Fig. 3B). Nevertheless, when the seeds are transected, low GFP signals can be distinguished inside the disinfected viable seeds (Fig. 3A, Fig. 6B), but they are hard to contrast with the negative controls. With a PathoScreen analysis, a small difference in the relative fluorescence intensity between disinfected seeds and the negative controls were found (Fig. 6A). Transected and not disinfected seeds showed a high fluorescent signal in the seed coat and trichomes, also in the internal tissues after 5 days of incubation (Fig. 3A, Fig. 6B). This observation is confirmed by measuring the relative fluorescence intensity in these seeds with the PathoScreen (Fig. 6A).

In dead seeds, inoculated with the GFP-Cmm strain and observed with a PathoScreen system, a high GFP signal could be found on seed coat of those seeds after 120 hpi (Fig. 4) and measured (Fig. 5A). The colonization of the internal tissues could only be observed by CSLM, where single cells could be spotted in the endosperm at 3 hpi. After 120 hpi, a massive colonization of the endosperm and embryo was found (Fig. 7).

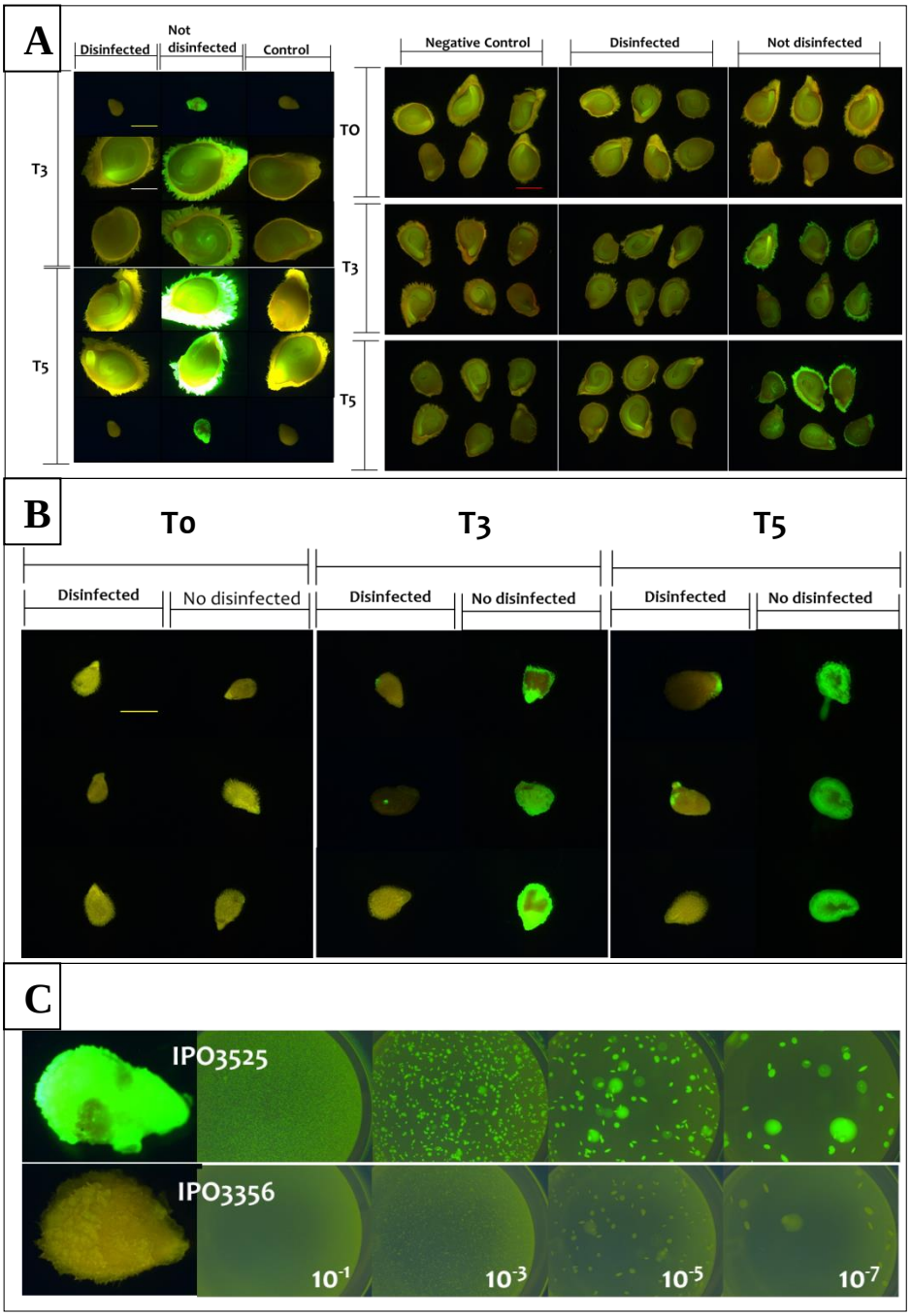
A
Disinfected
Not disinfected
Control
T3
T5
Negative Control
Disinfected
Not disinfected
TO
T3
T5
B
To
T3
T5
Disinfected
No disinfected
Disinfected
No disinfected
Disinfected
No disinfected
C
IPO3525
IPO3356
10-1
10-3
10-5
10-7

Figure 3. Epifluorescence stereomicroscope images of scarified tomato seeds vacuum-infiltrated with a GFP-tagged strain of *Clavibacter michiganensis* subsp. *michiganensis* or with sterile distillated water (control) and incubated on TBY medium (+antibiotics) for 0 , 3 or 5 days at 25^{o}C. Vacuum-infiltrated seeds were disinfected with 70% ethanol or untreated before incubation on blotters. (Yellow scale bar – 6 mm; White scale bar – 3mm; Red scale bar – 2mm). (A) Transected tomato seeds; (B) Undivided tomato seeds; (C) GFP Fluorescence shown on tomato seeds inoculated with a GFP – tagged strain of Cmm (IPO3525) or its parental (IPO3356) and its referent dilution plated in TBY^{+} media.

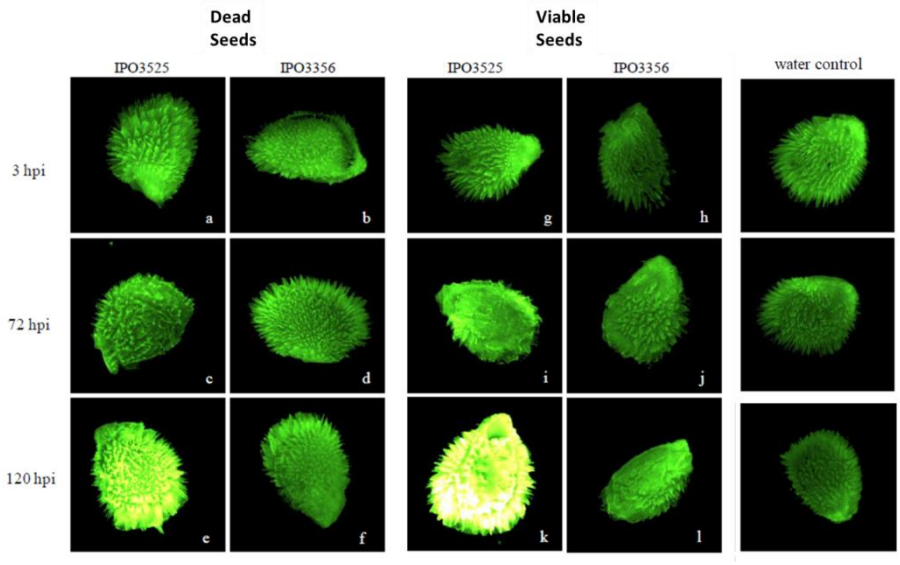

Figure 4. PathoScreen analysis of viable and dead tomato seeds inoculated with *Clavibacter michiganensis* subsp. *michiganensis* at 3, 72 and 120 hours post-inoculation. (a-f) Dead seeds; (g-l) Viable seeds. Seeds were inoculated with the Cmm GFP – tagged strain IPO3525 or its parental strain IPO3356 with a density of ca. 10^8 cfu mL^{-1}. Water control was inoculated with sterilized distillated water.

The PathoScreen system detected a high fluorescent signal, which likely originated from the seed coat after 5 days of incubation on TBY^+. Fluorescence measured with the PathoScreen increased in time in both dead (figure 4a-f) and viable tomato seeds (figure 4g-l) inoculated with the GFP-tagged strain IPO 3525, but not in seeds inoculated with the non-fluorescent parental strain IPO3356 or with water (negative control). Comparisons with the relative fluorescence intensity from dead and viable seeds showed no significant difference between the values (Fig. 5A-B), but when transected and undivided seeds are compared (Fig. 5, Fig. 6), a less fluorescence intensity is observed in the transected seeds. This probably occurs because, with an open seed, auto-fluorescent tissues are

exposed, and this auto-fluorescence is deducted from the fluorescent signal emitted by the GFP – tagged Cmm strain.

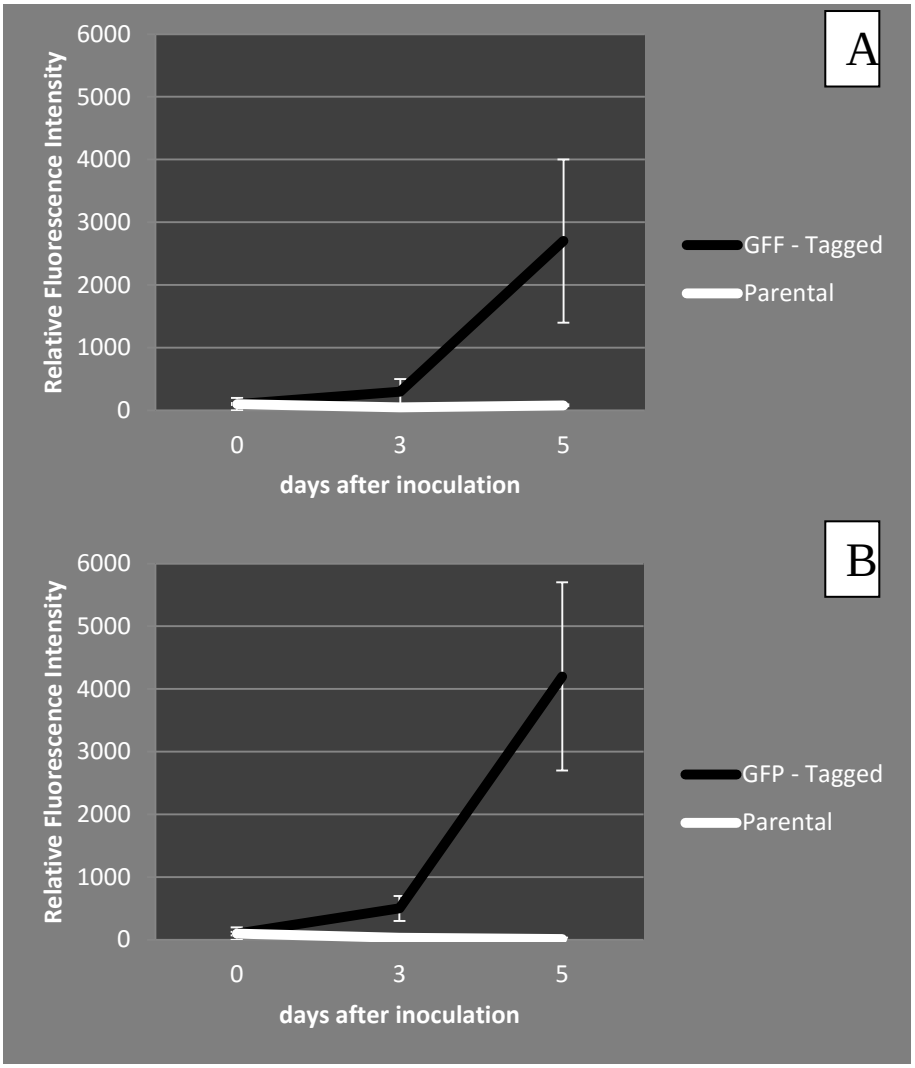

Figure 5. Corrected GFP signal from PathoScreen analysis of tomato seeds inoculated with the GFP-tagged strain IPO3525 of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) or its parental strain IPO3356

with a density of ca. 10^8 cfu $mL^{-1}$and incubated of seeds on TBY^+, a semi-selective medium for Cmm. The relative fluorescence intensity was determined on seeds killed by freezing and thawing (A), and on viable seeds (B).

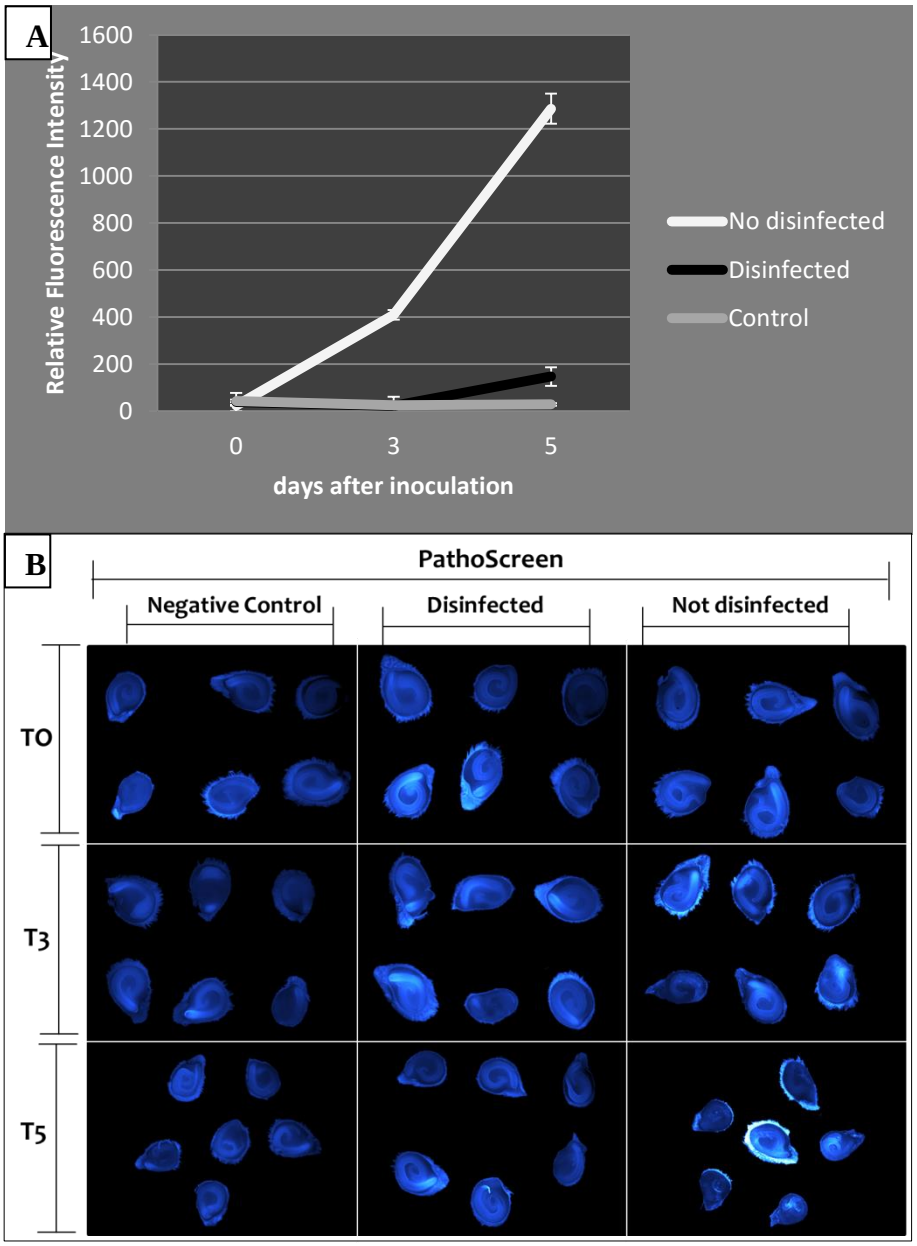

Figure 6.(A) Corrected GFP signal from PathoScreen analysis of viable tomato seeds inoculated with the GFP-tagged strain IPO3525 of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) with a density of

ca. 10^8 cfu $mL^{-1}$and incubated of seeds on TBY + antibiotics, a semi-selective medium for Cmm. The relative fluorescence intensity was determined on seeds vacuum-infiltrated but not disinfected, seeds vacuum-infiltrated and disinfected with 70% ethanol for 1 min and on seeds not vacuum-infiltrated with Cmm (negative control). (B) PathoScreen images of viable tomato seeds inoculated with *Clavibacter michiganensis* subsp. *michiganensis*. Seeds were inoculated with the Cmm GFP – tagged strain IPO3525with a density of ca. 10^8 cfu mL^{-1}.Seeds were vacuum-infiltrated but not disinfected, vacuum-infiltrated and disinfected with 70% ethanol for 1 min and or not vacuum-infiltrated with Cmm (negative control). All seeds were incubated on TBY medium (+antibiotics) at 0 (T0), 3 (T3) and 5 (T5) days after inoculation at 25°C.

With the CSLM analysis, at 3 hours post inoculation, some individual cells of the GFP-tagged transformant were detected in dead seeds (Fig. 7d). Cells may have been infected seeds during vacuum-infiltration, but smearing during transection of seeds, resulting in a translocation of cells originally present on the seed coat, cannot be excluded, even though seeds were effective washed in sterilized

distillated water before they were cut and placed in the microscopy glasses. At 3 dpi, bacteria were detected in the endosperm cells (figure 7e) and at 6dpi, a massive colonization of cells in the endosperm and embryo was found (figure 7f).

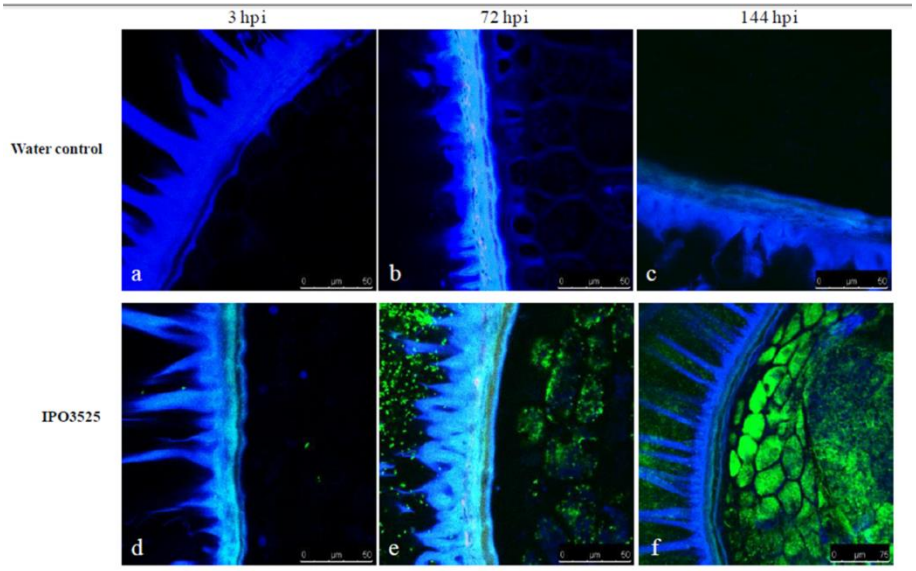

Figure 7: Images of dead tomato seeds inoculated with a GFP – tagged strain of Cmm analyzed by confocal laser scanning microscopy at 3, 72 and 144 hours post-inoculation. (a-c)Negative control seeds inoculated with sterile distillated water; (d-f) Tomato seeds inoculated with Cmm GFP - tagged strain IPO3525 with a density of ca. 10^8 cfu mL^{-1}.

In a repetition of the experiment, done in an exactly same way (but without any pre-treatment of the seeds), for unknown reasons, the

infection developed in a slower rate (data not shown). This might have occurred because internal infections with artificially infected seeds are hard to get when natural seeds are used, instead of those pre-treated (scarification or softening of the seed tissues) seeds. At 3 hours after colonization, no fluorescent cells were detected. At 6 dpi, low densities of fluorescent cells were detected in trichome hairs and intracellularly in the endosperm of dead seeds. At 8 dpi, the last sampling date, intracellular infections of the endosperm were detected (data not shown).

CSLM images obtained from the viable and pre-treated seeds (scarified and half disinfected after inoculation) (Fig. 8) showed cells of Cmm in the tissues of embryo and endosperm, in the disinfected and not disinfected seeds, right after the vacuum-inoculation with the GFP-Cmm suspension. After 3 days of incubation, an increase of the Cmm population can be seen in the tissues of both disinfected and not disinfected seeds, which cells migrate from the embryo and endosperm to other seed parts, like the cotyledons and radicle tip. Next, at 5 dpi, the Cmm population started to consume cells of the endosperm and embryo and grow in the seed coat, even in the disinfected seeds, clearly demonstrating the capacity of Cmm to enhance its population from a few

cells to high levels of densities inside of the tomato seeds after incubation on proper substrate (Fig. 8). In all seed samples tested, more than 80% of the seeds analyzed in the microscopy showed the presence of the GFP – tagged strain of Cmm (data not shown).

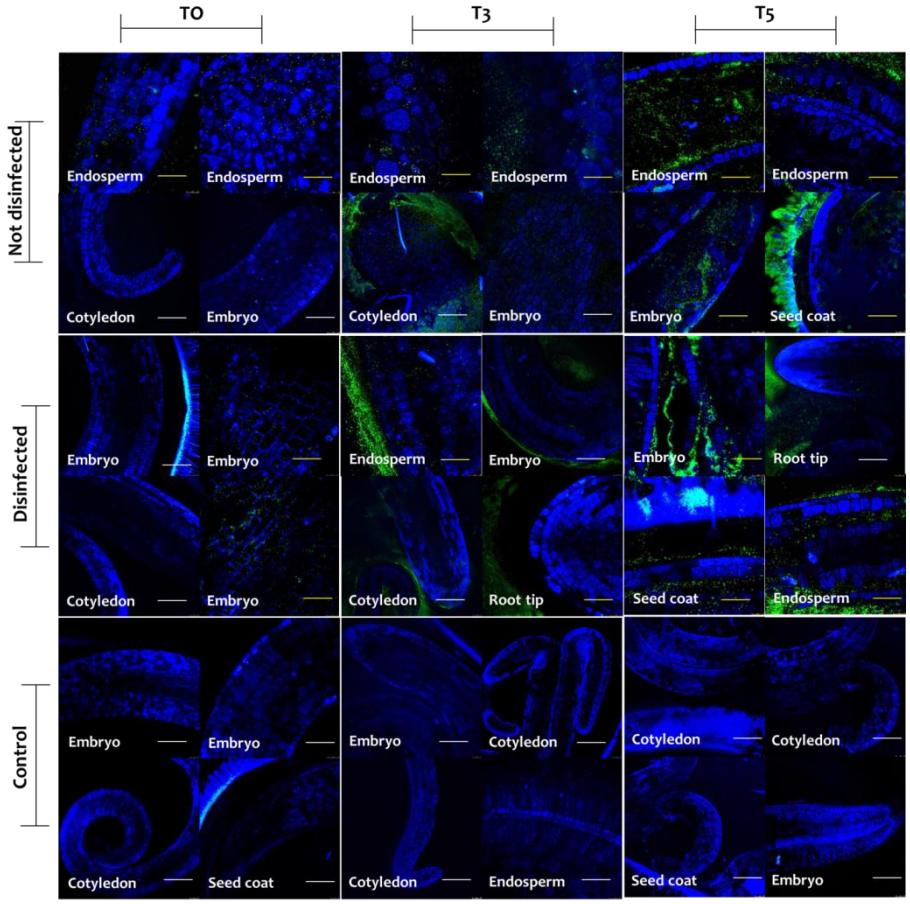

Figure 8. Confocal laser scanning microscope images of viable (transected) tomato seeds. Scarified seeds were vacuum-infiltrated with a GFP-tagged strain of *Clavibacter michiganensis* subsp. *michiganensis* or

with sterile distillated water (control) and incubated on TBY medium (+antibiotics) for 0, 3 and 5 days at 25^{o}C. Vacuum-infiltrated seeds were disinfected with 70% ethanol or left untreated before incubation on TBY medium (White scale bar – 100 µm; Yellow scale bars – 40 µm).

Seed extracts from the viable seeds inoculated on TBY^{+} or on nutrient broth were tested with a TaqMan assay to confirm the capacity of the qPCR probe to detect Cmm in seeds. Within the samples, all those that showed a Cmm population higher than ca. 10^{4} cfu seed^{-1} were certified as positive (data not shown). In those seeds which Cmm is found only in low internal incidence (disinfected seeds), 5 days of incubation on a semi-selective medium is required for a positive result with the TaqMan. In not disinfected seeds, immediately after the inoculation, positive results were obtained with the TaqMan test. To confirm the sensitive of the TaqMan probe with seed extracts spiked with a Cmm suspension, several tenfold dilutions were tested and the results observed showed that densities higher than ca. 10^{4} cfu seed^{-1} are required to positive results (Fig. 9).

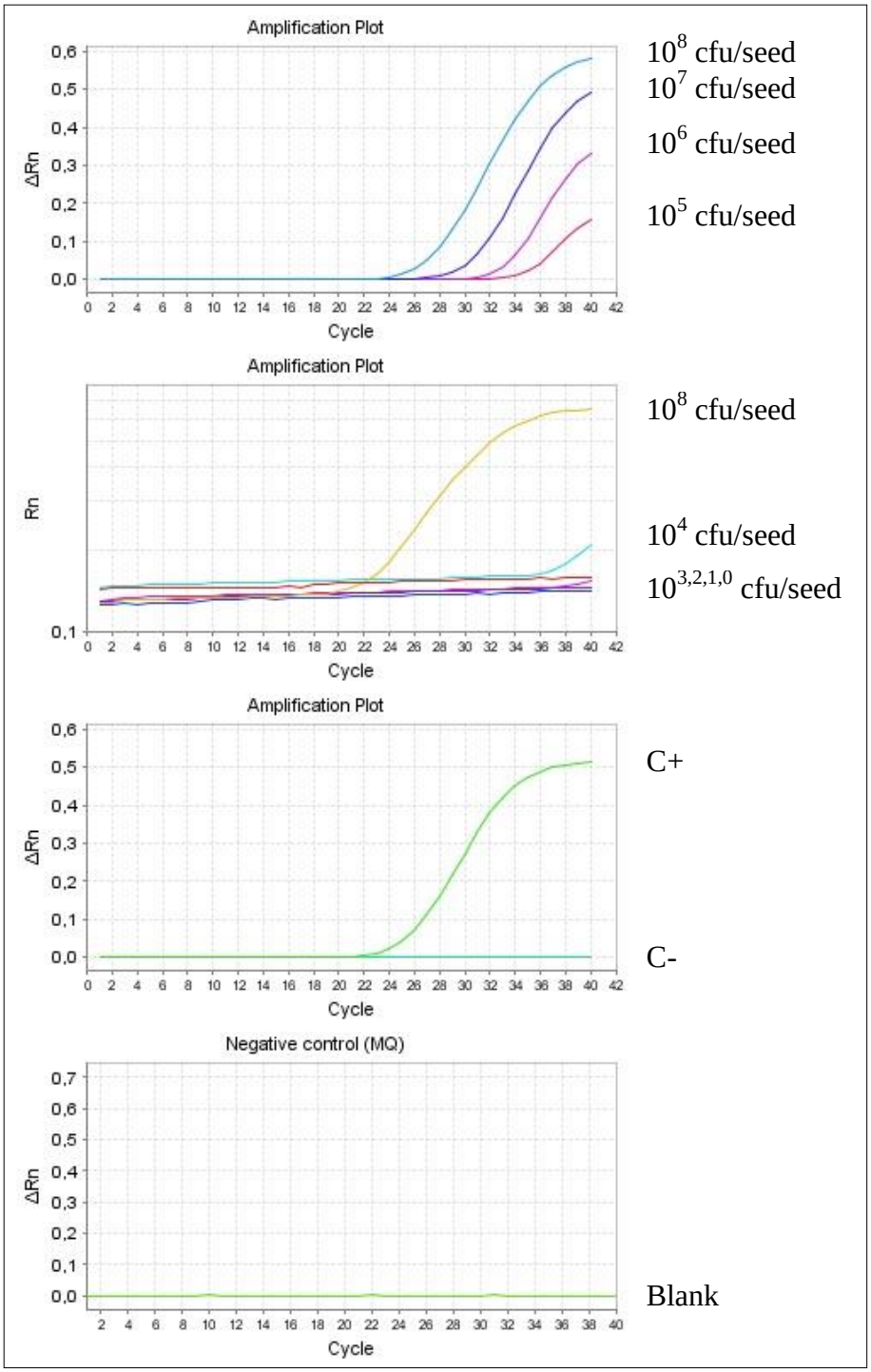
Amplification Plot
ΔRn
Cycle
10^8 cfu/seed
10^7 cfu/seed
10^6 cfu/seed
10^5 cfu/seed
Amplification Plot
Rn
Cycle
10^8 cfu/seed
10^4 cfu/seed
$10^{3,2,1,0}$ cfu/seed
Amplification Plot
ΔRn
Cycle
C+
C-
Negative control (MQ)
ΔRn
Cycle
Blank

Figure 9. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in seeds samples spiked with a GFP – tagged strain (IPO3525) using a TaqMan assay. Seeds were grinded in Ringer's solution containing several tenfold dilutions of GFP-Cmm. Positive Control (C+) is a sample of seeds vacuum-infiltrated with GFP-Cmm and incubated in TBY Medium for 5 days at 25°C. Negative control (C-) is a sample of seeds inoculated with sterile distillated water and incubated in TBY Medium for 5 days at 25°C. Negative control (MQ) is the blank for the qPCR reaction.

Discussion

Vacuum infiltration of tomato seeds (viable or dead) with Cmm followed by incubation of seeds on a Cmm selective medium (TBY) results in a strong increase of Cmm populations associated with seeds. Dead and viable seeds were used to observe and understand the behavior of Cmm in these types of seed tissues. The parental Cmm strain IPO3356, however, initially increased in densities up to ca. 10^{11} cfu mL^{-1} after 72 hpi, but then decreased to a density of ca. 10^{3} cfu mL^{-1} at 120 hpi. This fluctuation in the levels of seed population seems to occur quite often, as

it has already been reported before, where Cmm densities on pre-softened seeds dropped after 5 days of incubation on wetted blotters (Ribeiro et al., unpublished data). Further investigation to discover the real events that lead bacterial population to increase its own densities inside the seeds and then decrease after a certain amount of time it is needed. This fact could be explained by growth of other microorganism that could act as competitors or antagonists in biological control, extinguishment of the nutrients provided by the seed cells, or auto-regulating processes, like quorum sensing, that instigate the pathogen to produce toxins to regulate its own population in order to preserve the host and so to preserve itself, because dead seeds will not provide a suitable host where the pathogen can still perpetuate its cells and live on the field (Waters and Bassler, 2005).

An early study showed that seedlings grown from naturally infected seed can carry 10^3 to 10^4 CFU/g of the pathogen (Shirakawa et al., 1991) while seedlings grown from artificially infested seed may contain 10^4 to 10^8cfu/seedling (Tsiantos, 1987). With the process of enrichment, the Cmm population can reach, after a short incubation time, the titers higher than 10^{10}cfu seed^{-1} and more easily express the symptoms

and contribute to epidemiological and detection studies. Internal infections, mandatory required for seed treatment or detection studies, were also improved with seed incubation, attaining population densities of ca. 10^6 cfu seed^{-1} after 5 days.

Viable seeds that were inoculated and incubated to promote Cmm growth showed high levels of bacterial densities, even in just internal infections. Without enhancing, in previous study, 7 days after inoculation, the mean population of Cmm was about 10^8 cfu/cm of stem in symptomless transplants, where the Cmm population duplicated during this time. With this population titer, even after 17 days, lesions were not observed (Gitaitis et al., 1991). This might be explained for one decrease of population during the plant development or the utilization of resistant material (Coaker et al., 2004), because, as reported later, acropetal movement of Cmm in tomato plants resulted in an extensive systemic colonization of the whole plant reaching the apical region after 15 days (Chalupowicz et al., 2012), and so, symptoms development should be expected.

The systemic infection with populations of 10^8 CFU/g or higher leads to the characteristic wilting, stem canker and vascular discoloration

(Carlton et al., 1998; Gaterman et al., 2003; Meletzus et al., 1993). Before this, the pathogen can survive as an effective endophyte in tomato plants because, apparently, Cmm has to establish an endophytic population of at least of 10^8cfu/g of plant tissue in order to induce disease symptoms (Gaterman et al., 2003). As good endophyte, Cmm moves directly into the vascular tissue after its entrance through natural openings or wounds, which are easily made during grafting, clipping, harvesting, shipping and transplanting (Chang et al., 1991). Common characteristics involving systemic development and virulence among most of these vascular pathogens can be summarized in formation of extensive bacterial aggregates and biofilm attached to xylem surfaces, contribution of exopolysaccharide (EPS) to vascular blockage and symptom development and involvement of quorum-sensing (QS) in regulation of xylem colonization (Danhorn & Fuqua, 2007).

The use of a GFP-marked strain of Cmm allowed detailed studies on population dynamics and seed colonization. Other studies with tomato plants associated with a GFP – tagged Cmm strain already have been conducted, mainly on showing the movement of Cmm during the development of seedlings, in growing plants or fruits (Chalupowicz ate

al., 2012; Ribeiro et al., unpublished data; Tancos et al., 2013), but information about Cmm into seeds is poor or lacking. The GFP-tagged pathogen could be monitored using microscopic techniques and the PathoScreen system. Population densities could be measured with plating methods, and the presence of an extra antibiotic resistant marker (kanamycin) supported dilution plating techniques. As GFP was expressed in a stable way during the course of our experiments, the accompaniment of the Cmm behavior *in vivo* could be executed. At 8 dpi, fluorescent cells could still be observed with the microscopic techniques and at 5 dpi and 100% of the Cmm bacteria isolated from inoculated seeds highly expressed of GFP. This result confirmed previous studies with tomato plants on the stability of pK2-22 in strain Cmm382 (Chalupowicz et al., 2012) and Cmm IPO3356 (Lelis et al, 2014), which showed that the strain IPO3525 can still fluoresce after than 30 days *in planta*.

The microscopic work was done using viable and dead seeds. The performance of Cmm on these types of tissues was investigated. For dead seeds we proved that during seed incubation on TBY supplemented with antibiotics, Cmm populations increased on the seed coat (PathoScreen)

and in the seeds (CLSM), colonizing the endosperm and, after reaching high densities, the embryo tissues. It is supposed that, even without any improvement of its growth, Cmm still could grow in dead tissues, because of its ability for intracellular colonization by degradation of cellulose fibers from the primary or secondary cell wall using one of its major virulence factors, the endo-β-1,4-glucanases that cleave β-1,4-glycosidic bonds of these fibers internally (Jahr et al., 1999). Once inside of the cell, Cmm degrades starch reserves among others cell compounds for its nutrition (Gartemann et al., 2008; Labadie and Hébraud, 1997). This work showed that, even in dead seeds, Cmm still grows and these dead seeds could become an inoculum source in the field or in greenhouses because tomato seeds lots often do not show 100% of germination and dead seeds might be used during seedlings production (Ferreira et al., 2013; Shi et al., 2014).

On viable seeds, the pre-softening and scarification process allowed a larger number of Cmm cells to penetrate the seeds and start the colonization of embryo and endosperm. Untreated seeds (without pre-imbibition or scarification) hardly got internal infection after inoculation, and even if so, the bacterial population densities were too low to be

detected by qPCR technique and to be checked under GFP filters (data not shown). To circumvent this problem seeds were treated and the bacterial population was enhanced prior other tests. The scarification removed the trichomes and opened wounds on the seed coat and the imbibition created cracks on the surface of the seed, opening a way in for the bacterial cells (Ma et al., 2004). Next, densities of Cmm beginning to raise and cluster of fluorescent cells could be seen on the cotyledons, endosperm and embryo. After 5 days, damaged tissue could be observed indicating that, if the Cmm population does not start to regulate itself, probably the seed will not germinate anymore. However seedlings from artificially infected seeds still can be obtained (Ribeiro et al., unpublished data).

After colonizing viable seeds, infections of the embryo cannot be eliminated and will possibly result in a systemic infection, because the pathogen cannot be fully eradicated without considerable seed damage and germination unviability (Grondeau and Samson, 1994). In the disinfected seeds, Cmm cells were more often visualized inside of the embryo cells. This intracellularly colonization pattern is more described in late stages of disease development, where histological observations

revealed that pathogen populations first develops within large intercellular spaces beneath the stomata (external contamination) or the bacterial cells were first observed within vessels of the large marginal fimbriate veins (systemic contamination). Then, after a few days, large populations can be seen within the xylem vessels and in the end the tissue collapses, widespread occur and masses of bacteria can be seen in the intercellular spaces and intracellular spaces in necrotic cells (Bryan, 1930; Carlton et al., 1998, Wallis, 1977). Normally, bacterial cells on the first stages of infection remain on the intercellular space, but others plant bacteria have been reported on the intracellular spaces (Hogenhout & Loria, 2008; Joshi et al., 2007; Sharma et al., 2001). As occurs in artificially-infected seeds, Cmm cells infecting intact fruit cells, with intracellular colonization of pericarp cells consistently observed, has already been reported (Tancos et al., 2013).

Population dynamics to be studied in viable seeds with confocal scanning laser microcopy should not germinate and with the incubation of the seeds to stimulate the bacterial growth, the seeds should start the germination. However in thus study this problem was solved with the use of a high concentrated media or nutrient solution or with the use of

antibiotics. This condition might be due by the fact that with high concentrations of nutrient solution, the osmotic potential of the solution affects the seed germination and seedling fresh weight because seed germination damages is attributed to various factors involving high salt concentration affecting structural organization of proteins and alteration in stored reserves mobilization. Beyond that, seeds require higher amount of water uptake during the germination under salt stress due to the accumulation of the soluble solutes around the seeds which increases the osmotic pressure. With high concentrations of nutrient solution there is reduction in water availability, causing excessive uptake of the ions which results in toxicity in the seeds. Moreover, water potential gradient (reduced water availability) between the external environment and the seeds also inhibits the primary root emergence (Demir and Mavi, 2008; Falleiro et al., 2010; Machado et al., 2008; Zhang et al., 2010). The antibiotic Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms including plants, by interfering with the translocation step in protein thus blocking translational elongation (Schneider-Putsch et al., 2010).

PathoScreen analysis also confirmed the efficiency of Cmm to colonize tomato seeds, as an epiphytic bacteria on the seed coat and trichomes, or infesting the internal tissues like the endosperm and embryo acting as pathogen. Through the visualization of the fluorescence intensity increase over in time, independent of the condition of the seed, dead or viable, a Cmm population could be seen growing from a few single cells that are unable to be detected with plating methods or molecular biological tools, to a large population capable to cause epidemic diseases on the field. This system can also be used to monitor pathogens in several plant parts without destroying them and quantify the disease when the pathogen is associated with lesions, allowing automated scoring, independent of visual observations and manual scoring, thus excluding human error (Kastelein et al., 2014).

Furthermore, these results showed that one seed is able to carry high densities of the pathogen that can be used as an inoculum source within the producing areas. It was also observed that the fluorescence intensity in the experiment with undivided seeds was at least three times stronger than transected seeds at 120 hpi, although the amount of initial inoculum used was the same for both experiments. This fact could be

explained due the presence of auto-fluorescent tissues inside of the seeds, and when they are cut and exposed, the values of the auto-fluorescent intensity must be deducted from the values provided by the GFP signal, however the occurrence observed remains the same, that through time, Cmm is apt to enhance its population in seeds. We conclude that for capturing the signal in the PathoScreen relative high densities are required, higher than 10^9cfu seed^{-1} for dead seeds and 10^4 cfu seed^{-1} for viable seeds. Producing of the green fluorescent proteins might be affected by the Cmm growth in dead tissues, where in a living tissue a brighter GFP signal it is more easily recognized by the PathoScreen system.

As is well known, contaminated seeds are the main source of epidemic diseases on tomatoes fields caused by Cmm and this pathogen can survive in seeds for long periods (Kaneshiro and Alvarez, 2003). The pathway used by Cmm to reach the seeds it is characterized by multiple entry routes. In the last 10 years, the exactly method of entry into the fruit, and then infect the seeds, from external sources was considered unclear (Medina-Mora et al., 2001), mainly because a few amount of information was available at that time about the mechanisms of Cmm

pathogenesis, its colonization of seeds and subsequent transmission to seedlings. This is largely attributable to a lack of tools and difficulties in genetically manipulating this Gram positive bacterium (Metzler et al., 1997). One of the first studies on bacterial canker and wilting caused by Cmm tried to check the systemic movement of the pathogen until the seeds, however, no detailed histopathological studies have clearly demonstrated this natural route of seed infection (Bryan, 1930). Consequently, there was a need to develop more molecular studies that could allow a better understanding of Cmm infection process (Xu et al., 2010).

Nowadays, it is known that active penetration of Cmm occurs via seed scars formed when the seed is separated from funiculus or after subsequent infection of fruit lesions, followed by translocation of the pathogen via the mesocarp and the vascular system of fruits tissues into the seeds. Systemic infection of seeds can occur via colonization of the xylem vessels of peduncles and then reaching the seeds in formation (Medina-Mora et al., 2001; Tancos et al., 2013; Uematsu et al. 1977). In these systemic infections, Cmm can also penetrates the plant via root hairs (Ribeiro et al., unpublished data) which can be considered one

natural opening source of contamination, as was already reported for other pathogens of tomato plants (Bryan, 1930; Getz et al., 1983). Once inside of root and stems, the contamination follows all the tissue length, indicating probably the xylem vessels contamination, which may results in contaminated seeds during the fruit development (Tancos et al., 2013). Since Cmm is a systemic pathogen and extensively colonizes the lumen of xylem vessels and preferentially attaches to spiral secondary wall thickening of the protoxylem (Chalupowicz et al., 2012), it is hypothesized that it accesses the growing seeds by the host vascular system (De Léon et al., 2011).

Regarding to fruit infections, tomato fruits does not possess any natural opening like stomata, but symptoms development can be seen in fruits without wounds (Medina-Mora et al., 2001). The fact that may clarify this is that the pathogen can access the fruit by glandular and nonglandular trichomes that cover developing green fruit. Those trichomes starts to fall from the fruit when this begins to grow, which exposes open trichome bases and provides points of entry, as already reported for *Pseudomonas syringae* pv. *tomato* (Bryan, 1930; Getz et al., 1983).

Independent if Cmm attains the seeds by systemic or external routes, it was observed, in developing tomato seed, Cmm cells within the endosperm and funiculus (part of embryo), even if at relatively low levels. Beyond the fact that can actively access seeds systemically through the xylem, externally routes such through tomato fruit lesions or penetrating the ovary wall or floral parts are also used. The lesions harbor high intra- and intercellular populations, which, once the fruit began to ripen, present a translocation through the fruit mesocarp and nearby xylem vessels, than reaching the growing seeds (Tancos et al., 2013). After seed infection, Cmm moves to the seedling when the germination begins and during radicle protrusion, contact infections occur from the heavily infested seed coat. Incidentally, colonization of xylem vessels indicates systemic infections suggesting that the bacteria can move inside the vascular tissues after the seed germination and grow in the stems causing the bacterial canker, but a longer time than 7 days it is required to the bacterial population reach the seedling root (Ribeiro et al., unpublished data). Normally, Cmm is transmitted at low rate from seed to transplants when sowing infested seeds in greenhouses and transplanting the seedlings to the field (Chang et al., 1992). This way, asymptomatic

seedlings are another major cause that leads to epidemics disease spreading on the field by introducing inoculum sources.

The situation that into the seeds Cmm populations are found in relative small numbers highlights the difficulty in detecting and eradicating small initial pathogen populations within seed lots. Still, once the populations reaches transplant production or the field, exponential growth and rapid spread will occur because the pathogen can ooze from cankers and hydathodes, and in combination with rain and wind, Cmm spreads to distal leaves, fruit, and surrounding plants, and with this, visual symptoms will appear and damages will be caused. This infected seeds in combination with no external fruit or plant symptoms emphasizes the difficulty in identifying infected plants with potentially contaminated seed (Sharabani et al., 2013 P; Werner et al., 2002).

Cmm is largely spread on the tomato producing areas around the world and is internationally a quarantine organism (Anonymous, 2005). Thus, an accurate detection is a crucial step in confirming the presence and preventing the spread of bacteria to areas there are free of the pathogen. For this methods there are requirement for a fast, sensitive, highly specific, cheap and easy technique than can be used for

laboratories that certify seed lots. Different methods for detection have been described and each with their own advantages and disadvantages. Unfortunately there is no method that can gather all requirements and depending of its application, different methods or combinations of methods must be used (Sen et al., 2013).

Normally, three different types of methods for Cmm detection are in use: serological, DNA based and plating. Serological methods have a high risk of cross reaction with non-target organisms and the sensitivity of this method is low. Dilution plating on selective media is very sensitive but laborious, it takes 5–7 days to get results, requires lots of hand labor and in addition, confirmation of the nature of colonies is needed by other methods. This technique is the advised method by the International Seed Test Association and has been used for decades to identify and quantify Cmm (Fatmi and Schaad 1988). DNA based methods, like TaqMan real time PCR assays, are fast, sensitive and highly specific and it also allows quantification (Berendsen et al., 2011). TaqMan assay has been already used to identify *Clavibacter* subspecies (Bach et al. 2003) and to identify Cmm in seeds (Zhao et al. 2007). DNA is a relatively stable molecule that can persist for a long time in the environment upon cell death. So, even if

large amounts of DNA molecules are present in the seed samples, the TaqMan assay is not able to distinguish dead from viable bacteria. To avoid false positives, an enrichment step prior to detection to stimulate a few cells that might still present in the samples to grow to a higher concentration would prevent this problem. This enrichment process could be first plating the extract on a selective medium or on a selective broth prior to TaqMan (Bio-TaqMan assay), thereby, only viable bacteria that has increased in the seed samples will be detected. In this work we showed with these enrichment steps that a few number of cells can be enhanced to detectable levels.

Some ways to distinguish dead from viable Cmm cells has been used, like using the DNA binding dye ethidium monoazide (EMA). DNA from dead cells can be selectively removed during extraction by adding this dye to the samples. EMA penetrates only dead cells with a comprised membrane, binds to its DNA and is covalently linked to the DNA with light. During the extraction the DNA–EMA complex will precipitate whereas the unbound DNA remains in solution. EMA was effective up until a concentration of 10^8 cfu $mL^{-1}$of bacteria (Luo et al. 2008). However, this method demands that the Cmm population, after the DNA-

EMA removal, still can be detected even if presents in low levels. But normally Cmm is detected on seeds harvested from systematically infected plants in titers about $3x10^2$cfu/seed (Chang et al., 1992), which may be not impossible, but likely not enough, to be detected with rapid methods.

Sen et al. (2013) developed for the first time an indirect TaqMan real time PCR to identify and quantify Cmm in plant. They used successfully an internal amplification control (IAC) to excluded false negative results which did not affect the sensitivity of the TaqMan assay. The detection level in this study was determined at a level of 10^3 cfu/mL. In our work, using the same methodology, but without the IAC controls, the detection levels were settled to higher than 10^4 cfu seed^{-1}. The lack of the IAC control might have influenced the sensitivity levels. Still, the sensitivity was sufficient to detect the relatively high densities present in the seed samples after the population enhancement prior to detection. A high correlation between Ct values in the TaqMan assay and the concentrations based on the dilution plating on selective media was found. Thus, this method of incubation in a diluted selective broth prior to detection can be helpful to laboratories or other researchers that aim to

improve detection of Cmm low levels in seed samples, cause detection threshold levels of Cmm in seeds samples are relatively high (Hadas et al., 2005).

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(VERSÃO PRELIMINAR)

CONCLUSIONS

The lack of tomato seeds internally infected with Cmm is a major obstacle in the development of diagnostic or epidemiological tests, studies on seeds treatment or on transmission of the bacteria by seed, because, to simulate the natural conditions in which the seeds of tomato are infected with Cmm, they shall present internal infections, below the outer integument. The use of the technique that immerses seed lots in a Cmm suspension is an alternative that does not represent the natural conditions of infection, since the bacteria does not penetrate into the deeper tissues of the seed, and in turn these seeds are used for simulation in experiments using Cmm infected seeds. Infected seeds may also be generated by inoculation of tomato plants stems during the flowering, however, this technique is very expensive and the seed harvested from these plants only have Cmm populations on the external tegument of the seed. Our work described an inoculation method of tomato seeds with a Cmm suspension resulting in internally infected seeds, as clearly demonstrated by fluorescence microscopy. The germination of these seeds showed a transmission to the seedlings, which indicates that the seed can also be used in epidemiological studies. Seeds infected with the technique described in this study were also used to evaluate a detection method based on growth stimulation of internal Cmm populations in batch of seeds and has shown promising results. These initial experiments were performed in vitro on biosafety laboratories in the Plant Research International / Wageningen University in Wageningen, Netherlands,

because in this part of Europe this pathogen is still quarantine. As Cmm is not a quarantine bacteria in Brazil, but still of great economic importance, it is possible to continue the experiments in greenhouses to keep following the path of this bacteria in artificially infected seeds to seedlings and then to tomato plants, observing the development of characteristic symptoms of the disease, the future reisolation of Cmm in fruits and seeds and evaluation of fruit production in plants generated by the seeds internally infected.