Mapping tracks of *Xanthomonas campestris* pv. *campestris* resulting in Brassica seed infections

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Report of a topsector project 'Mapping tracks of *Xanthomonas campestris* pv. *campestris* resulting in Brassica seed infections' (2016-2020) (KV1505 095)

WR is part of Wageningen University & Research, the collaboration of Wageningen University and Wageningen Research Foundation.

Wageningen, December 2020

Report WPR-1028

Jan van der Wolf, Pieter Kastelein, Leo Poleij, Patricia van der Zouwen, Marjon Krijger, Odette Mendes, Jan Bergervoet, Bernadette Kroon, Pauline Bernardo, Reindert Nijland, 2020. *Mapping tracks of Xanthomonas campestris pv. campestris resulting in Brassica seed infections.* Wageningen Research, Report WPR-1028. 58 pp.; 19 fig.; 21 tab.; 55 ref.

This report can be downloaded for free at https://doi.org/10.18174/536441

In this report research is described, conducted by Wageningen Plant Research (Wageningen University and Research) in collaboration with the seed companies Bejo Zaden b.v., Syngenta Seeds b.v., ENZA Zaden Research and Development b.v. and Rijk Zwaan Zaadteelt and Zaadhandel b.v. within the frame of public private partnership project entitled 'Routes van *Xanthomonas campestris* pv. *campestris* die resulteren in zaadinfecties in Brassica in kaart brengen' (Mapping tracks of *Xanthomonas campestris* pv. *campestris* resulting in Brassica seed infections).

The project in Netherlands was granted by the Topsector 'Horticulture and propagation material' of the Dutch Ministry of Economic Affairs. It was conducted under grant number KV1505 095.

Key words: pollen infection, systemic colonization, green fluorescence protein tagged strains, fluorescence microscopy, confocal laser scanning microscopy

Report WPR-1028

Photo cover: Black rot in cabbage caused by *Xanthomonas campestris* pv. *campestris*

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Preface

In this report the results are laid down of the PPS project 'Mapping tracks of *Xanthomonas campestris* pv. *campestris* (Xcc) resulting in Brassica seed infections' (KV1505 095), conducted in the period from 2016 to 2020. Seed companies experience unexplained infections of their Brassica seed with Xcc, the causative agent of black rot. It has been evidenced that the risks for seed infections after inoculation of reproductive organs is high. This project was initiated to assess the risks for seed infections via the vascular route after inoculation of leaves or peduncles. In addition, the risks for contamination of pollen with Xcc was assessed. To allow detailed microscopy studies on the colonization of plant tissues, we generated and used Xcc strains tagged with a green fluorescent protein.

We like to express our gratitude to the seed companies for their support to the project, not only financially, but also by executing experiments, by giving advises and providing plant material. We thank the Topsector 'Horticulture and propagation material' of the Dutch Ministry of Economic Affairs for their coordination and financial support.

Summary

Xanthomonas campestris pv. *campestris* (Xcc) is a seed-borne bacterial plant pathogen causing black rot in Brassicaceae species. Seed companies experience unexplained infections of their Brassica seed with Xcc. It has been already evidenced that the risks for seed infections after inoculation of reproductive organs is high. This project was initiated to assess the risks for seed infections via the vascular route after inoculation of leaves or peduncles. In addition, the risks for contamination of pollen with Xcc was assessed. In chapter 1, studies on the potential role of pollen in the epidemiology of Xcc were conducted. After inoculation of the rachises, 2.9-5% of the anthers carrying on average ca. 10^4 pollen per anther were found infected with Xcc. Likely the pathogen had moved up via xylem vessels present in the filament into the anther which may have resulted in contamination of pollen. In chapter 2, Xcc strains were generated with a stable expression of the green fluorescent protein (GFP) to visualize Xcc *in planta*. A vector was constructed for chromosomal integration of reporter genes via both single and double cross over at the locus of the native secreted alpha-amylase gene. Using this vector we established expression of sGFP driven from the alpha-amylase promoter. In chapter 3, translocation of *Xanthomonas campestris* pv. *campestris* (Xcc) in *Brassica oleracea* var. *botrytis* vernalization-independent cauliflower plants was studied to investigate the pathways of the pathogen via infected leaves into the seed. After inoculation of petioles, spray-inoculation of young plants, inoculation of rachises or inoculation of pedicels, the colonization of plant tissues was examined using dilution-plating. Studies with pedicel-inoculated plants were conducted with green fluorescent protein (GFP)-tagged strains to allow also localization of the pathogen with fluorescence microscopy techniques, i.e. epifluorescence stereomicroscopy (ESM), fluorescence microscopy (FM) and confocal laser scanning microscopy (CLSM). After inoculation of petioles, no translocation to the stems was found as a rapid senescence of the inoculated leaves was found, resulting in leaf fall. Spray-inoculation of young plants of both cultivars in the six-leaf stage caused a severe symptom development. Translocation of Xcc in the developing plants was found to the top of the peduncles. The pedicels of infected peduncles, however, were all negative, indicating that the translocation of Xcc in spray-inoculated plants was limited to the peduncles. Inoculation of rachises resulted frequently in symptom expression on rachis and siliques. Using a dilution plating assay, infections were found in the inoculated rachises, pedicels, siliques, septa and in the seed, both externally (seed washings) as internally (seed extracts after disinfection). However, only rarely a fluorescent signal was found on siliques or seeds using epifluorescence stereomicroscopy (ESM). Inoculation of the pedicels resulted in a high infection incidence of the siliques. Using FM and CLSM, bacteria were found mainly in vascular tissue and the parenchyma cells of the replum of the siliques. Incidentally, Xcc was located in the septum or found in biofilms attached to the endocarp. In conclusion, we found that after spray-inoculation, Xcc could migrate from spray-inoculated leaves of young plants into the distal end of the peduncles. Although population densities in the peduncles could be high, no infections in the pedicels were found. Possibly, migration of Xcc into peduncles and/or the development of populations in peduncles was too late for migration into pedicels. If high densities of Xcc were inoculated in the rachises of peduncles, Xcc was able to migrate into the pedicel nearby the inoculation point and further into siliques and seeds with a high efficiency, even resulting in deeper seated seed infections. Studies with fluorescence microscopy indicated that after pedicel inoculations Xcc migrated via the vascular bundle of the suture. Seeds may have become infected via vascular tissue in septum and funicle, but also via contact with Xcc growing as a biofilm on the endocarp of the valves of the siliques.

1 Potential role of Brassica pollen in dissemination of *Xanthomonas campestris* pv. *campestris*

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Abstract
The role of pollen of *Brassica oleracea* plants in the dissemination of *Xanthomonas campestris* pv. *campestris* (Xcc) is unknown. In theory, pollen carrying the pathogen may contaminate the pistil during fertilization, which potentially can result in seed infections. In replicated experiments, we investigated if pollen could become contaminated after inoculation of the basis of peduncles or rachises just before flowers opened. It turned out technically impossible to collect pollen free of other plant tissues; in particular contamination of pollen with ageing petals could not be avoided. Therefore, instead, anthers carrying on average ca. 10^4 pollen per anther were collected after removing subsequently sepals, petals and style using sharp-pointed tweezers. Anthers were excised using dissection scissors. No infections of anthers were found after inoculation of the basis of the peduncles. After inoculation of the rachises, 2.9% of the anthers were found infected with Xcc in 2016 and 5% in 2017. Likely the pathogen had moved up via xylem vessels present in the filament into the anther which may have resulted in contamination of pollen.

Introduction
In several pathosystems, pollen have been recognized as carriers of bacterial pathogens. For example, *Xanthomonas manihotis* was detected in macerated pollen of cassava using an immunofluorescence assay and the authors concluded that seed infection could occur by a pollen-to ovule transfer (Elango & Lozano, 1980). *Xanthomonas juglandis* were detected in pollen from diseased catkins of walnut trees using dilution plating (Ark, 1944). It was also demonstrated that inoculation of catkins with a mixture of diseased and healthy pollen could result in symptomatic infections. The bacteria were well able to grow on agar jelly with no other nutrient than walnut pollen. Pollen were also found involved in the dispersion of *Pseudomonas syringae* pv. *actinidiae*, the causative agent of bacterial canker of kiwifruit (Vanneste et al., 2011, Stefani & Giovanardi, 2011).

Knowledge on the role of pollen in the epidemiology of plant pathogens is important for disease management. In the first place by prevention of contaminations, but pollen may also be treated against bacterial pathogens. A dry heat treatment of the pollen at 35 °C at a relative humidity of 50% or less for 20 h, killed the pathogen probably by a combination of heat and desiccation, without affecting pollen viability (Everett et al., 2012).

The aim of this study was to determine the potential role of pollen in the epidemiology of *Xanthomonas campestris* pv. *campestris*, causal organism of black rot in crucifers. We first determined if pollen can become infected with the pathogen after inoculation of peduncles or rachises just before flowers opened.

Materials and methods (2016-2017)
Bacterial strains and growth conditions. Xanthomonas campestris pv. *campestris* strain IPO3076 (working collection WUR) (Xcc) was stored at 17 °C on YDC plates (Duchefa Biochemie) for a maximum of one month. Bacteria used for inoculation were grown on TSA plates (Oxoid, CMO131) for 48 h at 25 °C. Isolation from plant tissues and pathogen quantification was done using mFS plates (Duchefa Biochemie). mFS plates were incubated for 5-7 days at 25 °C before counting the pale green, mucoid colonies surrounded by a small zone of starch hydrolysis, typical for Xcc. Suspensions were prepared in phosphate buffered saline (per liter: 8 g NaCl, 13.5 g $Na_2HPO_4.12H_2O$, 2 g $NaH_2PO_4.2H_2O$, pH7.2) or PBS supplemented with 0.01% Tween20 (PBST).

Plant material. Experiments were conducted in 2016 and 2017. *Brassica oleracea* var. botrytis nr 103598.1451 (Rijk Zwaan) a vernalisation independent cauliflower breeding line was used in both years. In 2016, seeds were sown in a seed tray with Horticoop seedling mix on 19 September. In 2017, seeds were sown on the 20th of September. Eight days after sowing, seedlings were transplanted in 8 cm pots (Pöppelmann TEKU), after 31 days in 12 cm pots and after 59 days in 3L pots with Horticoop potting soil. Plants were grown till inoculation at 18/15 ^{o}C (day/night) and from inoculation onward at 24/20 ^{o}C (day/night), and a relative humidity of 55-60%. Plants were fertilized weekly. Sciarid flies were controlled by restricted watering of plants and trapping the insects with yellow sticky traps (Koppert). Grey mould (*Botrytis cinerea*) was also prevented by restricted watering and by two applications of chlorothalonil (Daconil, 0.3%) on the curds.

Inoculation. One day before inoculation, plants were not watered; next watering was done shortly after inoculations. The basis of all peduncles per plant was inoculated with suspensions of ca. 10^7 cfu/ml, by diluting a suspension with an OD_{620} of 0.1 ten-times with PBS using a syringe and a 30G needle. In the experiment of 2016, the peduncle basis was inoculated at 87 days after sowing. In the experiments of 2016 and 2017, the rachises under sampled flowers were inoculated with 10^9 – 10^{10} cfu/ml (a ten-times concentrated suspension with an OD620 of 0.1) just before flowers start to open at 7 different occasions between 88 days and 106 days after sowing (Figure 1A).

Figure 1 *A. Rachis with inoculated lesions (14 dpi). B. Flower dissection. C. Filament with vascular tissue and pollen.*

Collection of airborne pollen around flowers. In the experiment of 2016, on two days pollen were collected around flowers of 6 clusters (plants grouped around the inlet of the filter holder) of 5 inoculated plants and 2 clusters of 5 mock-inoculated plants at 4 dpi, by a procedure similar as described previously (Johnson-Brousseau & McCormick, 2004). For this experiments, plants were used 11-13 dpi after leaf inoculation. Leaves were inoculated 66 days after sowing. On the first (sunny) sampling day, pollen were collected in the afternoon. On the second (cloudy) day, sampling was done early in the morning and shortly after midday. At the time of sampling plants were in full blossom, with both overblown flowers and unopened buds. We used a laboratory vacuum pump (KNF-Verder, NL), with Swinnex 25mm filter holder (Millipore), holding a 8µm isopore carbocarbonate membrane filter (Millipore). After a sampling time of 10 min using a maximum suction power, the membrane filter was transferred to the bottom of a 50 ml Greiner tube. Particles collected on the filter were eluted by vortexing for 2 min in 1.5 ml PBST. The suspension was transferred to a 2 ml-centrifuge tube and the particles were centrifuged for 10 min at 10,000 rpm (4 ^{o}C). One ml of the supernatant was removed and the particles resuspended in the remaining fluid. Hundred µl of the undiluted and the 100 times diluted concentrate was plated on mFS medium. From the concentrate, 25 µl was used for counting numbers of pollen by using a light microscopy (compound microscope, 5x objective and 10x ocular).

Collection of flower bases and anthers of peduncle-inoculated plants. In the experiment of 2016, at 4 dpi recently opened flowers were collected from 6 clusters (which is a group of plants with a similar stage of development) of 5 inoculated plants and 2 clusters of 5 mock-inoculated plants at 5 and 7 dpi. For each plant two flowers were cut off close to the junction of pedicel with the rachis. Flowers

were arranged in 24-wells plates, such that contact between flowers was avoided. Prior to dissecting the anthers from each flower, subsequently sepals, petals and style were removed using sharp-pointed tweezers (Figure 1). Anthers were excised using dissection scissors. Between each flower, dissection tools were disinfected in 70% ethanol. Flower bases (pedicel and receptacle) and anthers of the same cluster were collected in the same extraction bag (60 × 80 × 0.09 mm plastic bag) and 2 ml-Eppendorf tube, respectively. Flower bases were crushed in their extraction bag using a hammer and directly after crushing 1.5 ml of PBST was added. Anthers were crushed in their tube using the blunt side of a sterile disposable inoculation needle, after which 1.5 ml of PBST was added, which was followed by suspending the pollen using a vortex. Hundred µl undiluted and 100 times diluted suspensions were plated on mFS medium. Twenty-five µl of the 100x diluted suspension was used for counting numbers of pollen by light microscopy (compound microscope, 5x objective and 10x ocular).The growth of Xcc was classified as — (no growth of Xcc), + (<100 colonies), ++ (dense growth).

Collection of flower bases and anthers of pedicel-inoculated plants, In the experiments of 2016 and 2017, flowers were collected and dissected as described for the peduncle inoculated plants, but from inoculated rachises and mock-inoculated plants at 5 and 10 dpi. Of each individual flower the basis (pedicel and receptacle) and the anthers were checked for presence of Xcc as described above.

Results

Stab-inoculation of the basis of the peduncles did not result in infection of the flower basis (pedicels and receptacles) nor in infection of the anthers (Table 1). In contrast, inoculation of the rachises near to the sampled flowers resulted in an infection percentage of the flower basis of 49% and 44% in 2016 and 2017, respectively. Most of the these samples were highly infected with Xcc. The pathogen was also found in 2.9% and 5% of the anthers sampled in 2016 and 2017, respectively, but the densities were often low. The average number of pollen per anther varied between $1.1x10^4$ and $2.7x10^4$ of both years (Table 2).

Table 1 *Incidence of flower basis (pedicels and receptacles) and anthers contaminated with Xanthomonas campestris pv. campestris (Xcc) after stab-inoculation of peduncle basis.*

Inoculum	Anthers		Pedicels	
	N	Xcc-positive	N	Xcc-positive
PBS	4	0	4	0
Xcc	12	0	12	0

Table 2 *Incidence of flower bases (pedicels and receptacles) and anthers contaminated with Xanthomonas campestris pv. campestris (Xcc) after stab-inoculation of rachises.*

Treatment			Xcc-positive (%)		N pollen/anther	
	Year	N flower	Flower basis	Anthers	Average	St dev
Non-inoculated	2016	26	0	0	$2.7x10^4$	$1.5x10^4$
	2017	11	0	0	$1.1x10^4$	$8.1x10^3$
Inoculated	2016	103	49[a]	2.9[b]	$2.7x10^4$	$2.2x10^4$
	2017	100	44[c]	5[d]	$1.4x10^4$	$8.1x10^3$

[a] all but 3 samples with high densities of Xcc exceeding 100 cfu per flower basis.

[b] only 1 sample with high densities of Xcc exceeding 100 cfu per anther.

[c] all but 10 samples with high densities of Xcc exceeding 100 cfu per flower basis.

[d] 2 with high densities of Xcc exceeding 100 cfu per anther.

The use of a vacuum pump with an integrated filter to collect pollen free from other contaminants had a limited success. From the two mock-inoculated plants no pollen were trapped. From six Xcc-inoculated plants, pollen were found in only in two samples, one with 200 pollen and one with 19200 pollen. Xcc was not detected.

Discussion and Conclusions

Brassica is a cross-pollinator and the use of pollen from a crop in which Xcc is present bears a risk for carry-over contamination to plants and in particular a risk for pollen-to ovule transfer resulting in seed infections. Flower inoculations with Xcc have been shown to result relatively easy in seed infections (van der Wolf & van der Zouwen, 2010, van der Wolf et al., 2013, Kastelein et al., 2014).

Inoculation of rachises near to the sampled flowers with Xcc resulted in translocation of the pathogen to the pedicels, and through the filament up to the anthers, although with a low incidence of 3-5%. Likely the pathogen has moved up to the anther via the xylem vessels in the filament (Figure 1C). Infections may have resulted in infection of pollen as also xylem tissue is found in the anthers (www.yourarticlelibrary.com/reproduction-in-plants/structure-of-stamen-anther-pollen-sac-and-pollen-grain-in-plants-biology/26771). However, we were unable to proof this experimentally. We were unable to collect sufficient pollen free from impurities by using a vacuum pump connected to a filter to concentrate pollen from the air.

In our experiment, Xcc did not move from the base of the peduncles to the pedicels in a period of 7 days after inoculation. In experiments in 2019-2020, the pathogen was translocated from spray-inoculated, highly symptomatic leaves to the peduncles, but not to the pedicels, in a period of 60-80 days. These combined data indicate that translocation from leaves to anthers and seed through vascular tissue after inoculation of leaves or the basis of peduncles is expected to be a rare event. However, in our studies, a tropical variety of *Brassica oleracea* was used. It cannot be excluded that in Brassica crops which are grown biannually, and are flowering after vernalization, Xcc is more easily translocated to seeds after leaf infections. To proof this, plants should be grown under insect-free nets and pollination should be done manually, to avoid that seed become infected via infections of flowers.

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2 Chromosomal integration of reporter genes in the amylase locus of *Xanthomonas campestris* pv. *campestris*

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Abstract
Xanthomonas campestris pv. *campestris* (Xcc) is a seed-borne bacterial plant pathogen infecting Brassicaceae species. Information on colonization routes of the pathogen in plants resulting in seed infections is limited and there is obviously a need for tools to visualize Xcc *in planta*. Here, we present a vector for chromosomal integration of reporter genes via both single and double cross over at the locus of the native secreted alpha-amylase gene. Using this vector we established expression of sGFP driven from the alpha-amylase promoter. The GFP expression was stable for five generations, whereas a similar strain harbouring GFP on a replicating plasmid half of the colonies lost the GFP expression. The use of long read nanopore sequencing using the MinION sequencer enabled rapid and complete analysis of the way the plasmids were integrated in the genome and allowed us to explain the observed phenotypes. Using this newly engineered strain we were able to study its distribution in cabbage, showing strong colonization of the xylem and the leaves.

Introduction
Black rot of crucifers is caused by the seed-borne, Gram-negative bacterium *Xanthomonas campestris* pv. *campestris* (Xcc), a pathogen that can infect a wide range of plants within the crucifer family *Brassicaceae*, including all cultivated brassicas, but also Arabidopsis, a plant used in molecular model studies (Williams, 1980). The disease is characterized by blackened vascular tissues and foliar marginal V-shaped chlorotic or necrotic lesions (Cook et al., 1952b). As the disease progresses, vessels in the main stem turn black, and the plant becomes wilted, stunted and finally rots. The disease is found throughout the world and is one of the most destructive diseases of crucifers (Williams, 1980). The pathogen has a high degree of variability with respect to the virulence on host plants, serology, genetics, and with respect to physiological and biochemical properties (Alvarez et al., 1994; Dzhalilov & Tiwari, 1995).

Black rot epidemics easily occur by the rapid rates of pathogen multiplication and spread (Roberts et al., 2007). The pathogen, which once established, can multiply and spread fast if conditions are favourable for the pathogen, i.e. at a high relative humidity and temperatures between 25-30 ^{o}C. Infected seed can be the primary source of inoculum (Cook et al., 1952a; Krauthausen et al. 2018), but also debris of cabbage plants in or on soil (Dzhalilov & Tiwari, 1995; Köhl et al., 2011), refuse piles of cabbage (Kocks & Zadoks, 1996), or inoculum present on alternative host and non-host plants in the environment (Krauthausen et al., 2018; Schaad & Dianese, 1981). The pathogen can be dispersed by splashing water (Roberts et al., 2007), aerosols (Kuan et al., 1986), and possibly by insects (Shelton & Hunter, 1985; Van Der Wolf et al., 2010), but contact infections with contaminated machines, furs, feathers or clothes may also play a role in the dissemination (Williams et al., 1972, Kuan et al., 1986). During germination of infected seeds, the pathogen invades the mesophyll and the vascular tissues of the epicotyl, infects foliage and is released through guttation droplets at leaf margins. After bacteria are deposited on the leaf surface, the epiphytic populations may build up, such

as have been demonstrated for other Xanthomonads (Poplawsky and Chun, 1998). Plants are mainly invaded through hydathodes and occasionally via leaf and root wounds and other natural openings such as stomata and the openings at root junctions (McElhaney et al., 1998). It has been suggested that also flower infections, caused by pollinating insects play a role in the occurrence of seed infections (Van der Wolf & van der Zouwen, 2010, Van der Wolf et al., 2013).

In an infected seed crop, black rot symptoms may develop well, but frequently infected seed is also harvested from crops not showing visible black rot symptoms (Van der Heijden, Bejo, Warmenhuizen, the Netherlands, personal communication). Many questions remain on colonization routes of Xcc resulting in seed infection. Xcc can move through the infected pods via the funiculi into the seed (Cook et al., 1952a) and possibly via a direct contact between infected valves and developing seed (unpublished observations). From there, a further invasion of the seed coat, endosperm and embryo can be expected (Van der Wolf et al., 2019). In seeds with infected funiculi and seed coats, Xcc likely is able to resist chemical- and hot water treatments applied as seed treatments, but full evidence is lacking.

There is obvious a need for methods to visualize in detail the translocation of Xcc *in planta* that results in seed infection and also to visualize the localization of the pathogen in and on the seed. To be able to identify and localize the Xcc bacteria growing on and inside plant tissues the expression of a fluorescent protein can be of great help. Some fluorescent strains of Xanthomonas are reported in the literature (Cubero et al., 2011; Zhang et al., 2009). Unfortunately, the expression of *gfp* in these strains is based on replicating plasmids. These plasmids are often rapidly lost when selection pressure is absent (Sabuquillo et al., 2017; Van der Wolf et al., 2019). In this study, we set out to develop a strategy to facilitate stable chromosomal integration of gene encoding a superfolded green fluorescent protein (sGFP), with a fluorescence signal high enough to distinguish the bacterium from the background fluorescence of the plant material. Integration of a fluorescent reporter in the chromosome should prevent the loss of fluorescence in the absence of selection pressure.

We identified the gene encoding a secreted amylase as a suitable locus for chromosomal integration, and successfully integrated the marker in the Xcc chromosome. sGFP expression is controlled by the native amylase promoter, and was readily detected *in planta.*

Materials and Methods

Culturing of Xcc and E.coli
E.coli strains were cultured in LB medium at 37°C. Transformation of *E.coli* was done by chemical competence induction as described (Sambrook et al., 1989). Xcc strains were routinely cultured in Trypticase Soy Broth medium (Oxoid), both in liquid cultures as well as on solid medium where 1.5% agar was added. Deviating growing conditions are described below when applicable.

Creation of GFP integration vectors
To create GFP expression cassettes for targeted integration into Xcc strains we first assembled these in a vector with a pUC18R6K replicon, which only replicates in PIR-I strains of *E.coli* (Miller & Mekalanos, 1988). Plasmid pUC18R6K-mini-Tn7T was a gift from Herbert Schweizer (Addgene plasmid #64957) (Choi et al., 2005). This plasmid was isolated from its host strain, and digested with *PstI* (Promega). The other fragments were all generated by PCR using Phusion DNA polymerase flash mix (NEB) and primers and templates were as described below and indicated in tables 1A,1B and1C. The regions up and downstream of the alpha-amylase gene (NP_636139) as annotated in Xcc strain ATCC 33913 (NC_003902.1) were amplified by PCR using chromosomal DNA of Xcc strain IPO3076, and the obtained amplicons contain a linker overlapping the plasmid sequence and the downstream sequence. sGFP was amplified by PCR using plasmid pCM29 as a template, and primers introducing a linker sequence for Gibson assembly. The kanamycin resistance marker was amplified from plasmid pUC18R6KT-mini-Tn7-Km (Addgene plasmid #64969, (Choi et al., 2005)). The whole constructs were assembled using a one-step NEBuilder® HiFi DNA Assembly Master Mix (NEB) using a 5:1 molar ratio of inserts against the vector backbone, and subsequently the ligation products were transformed to chemically competent *E. coli* strain PIR-1 containing the empty vector. Colonies were selected on LB agar plates containing kanamycin (100 µg/ml). Clones were picked and isolated plasmids where

checked by restriction analysis and relevant parts were sequenced. The resulting vectors were transformed by electroporation to Xcc strains as described before (Amaral et al., 2005).

Table 1A *Primers for PCR.*

#	Name	Linker for Gibson assembly	Annealing sequence
1	AmyUp_F-puc18_link	CCAAGCTTCTCGAGGAATTCCTGCA	TGACCAGCACGCTGCGAATG
2	AmyUp_noP_R	-	GAATGGCGGCGGTGGTTGCCGGTA
3	AmyUp_RBS_R	-	ATCAGTGGACGCACGCATCCCGCACAGC
4	PsarA_F-amyUp_noP_link	ATACCGGCAACCACCGCCGCCATTC	CCAAATGCTAACCCAGAAAT
5	sGFP_RBS_F-amyUp_RBS_link	GTGCGGGATGCGTGCGTCCACTGAT	CCGGGTACCTTAGGAGGATGAT
6	sGFP_R-KmR_link	CTTGTCCAGATAGCCCAGTAGCTGA	TTATTTGTAGAGCTCATCCATGCCATGTGT
7	sGFP_R-AmyDown_start_link	AATCAACGTCGCCACATACGCGCCT	TTATTTGTAGAGCTCATCCATGCCATGTGT
8	KmR-F	-	TCAGCTACTGGGCTATCTGGACAAG
9	AmyDown-F	-	AGGCGCGTATGTGGCGACGTTGATT
10	KmR_R-amyDown_start_link	AATCAACGTCGCCACATACGCGCCT	CAGAGCGCTTTTGAAGCTGATGTG
11	AmyDown-R	-	CGGATGCGATCAGCGCTAAGACTTC
12	KmR_F-amyDown_end_link	GAAGTCTTAGCGCTGATCGCATCCG	TCAGCTACTGGGCTATCTGGACAAG
13	AmyDown_R-puc18_link	TCACTAGTGGATCCCCCGGGCTGCA	CGGATGCGATCAGCGCTAAGACTTC
14	KmR_R-puc18_link	TCACTAGTGGATCCCCCGGGCTGCA	CAGAGCGCTTTTGAAGCTGATGTG

Verification of genomic integration by nanopore sequencing

To verify how the plasmids had integrated in the Xcc chromosome we performed whole genome sequencing using Oxford Nanopore MinION. Genomic DNA was isolated using the phenol/chlorophorm extraction according to the method of Sambrook (Sambrook & Russell, 2006). Integrity was checked on a agarose gel. DNA of strains IPO4047 and IPO4048 (IPO3076_PsarA-GFP and IPO3076_Pamy-GFP) was pooled and a and a sequencing library was prepared using the 1D^2 LSK-308 kit according to the ONT protocol. The resulting library was sequenced on the MinION sequencer for 4 h. Reads were base called by Albacore integrated in MinKNOW v 1.7.3. To obtain a consensus sequence of the genomic region the GFP gene was integrated in, first all reads containing the sGFP gene were selected (169 from 52980 reads) using blast run from within Geneious V10.2.1 and reads were binned in hit / no hit. Next, the bin with *gfp* hits was again divided based on a blast search for the SarAP1 promoter, resulting in a bin containing 51 read with SarAP1 and a bin of 118 reads without the SarAP1 promoter. These two pools of reads were aligned and trimmed in Geneious, and a consensus sequence was computed using Canu v. 1.5. (settings: genomeSize=50k -nanopore-raw) (Koren et al., 2017).

Table 1B *Amplification scheme using primers from Table 1A.*

PCR PRODUCTS	Description	Template	Primers	Size (bp)
A	AmyUP_NoP (5' link to puc18)	Xcc strain IPO3076	1x2	736
A*	AmyUP_P-RBS (5' link to puc18)	Xcc strain IPO3076	1x3	971
B1	PsarA-RBS-sGFP (5' link to AmyUP and 3' link to KmR)	pCM29	4x6	1028
B2	PsarA-RBS-sGFP (5' link to AmyUP and 3' link to AmyDown)	pCM29	4x7	1028
B1*	RBS-sGFP (5' link to AmyUP and 3' link to KmR)	pCM29	5x6	795
B2*	RBS-sGFP (5' link to AmyUP and 3' link to AmyDown)	pCM29	5x7	795
C1	KmR (3' link to Amydown_start)	pUC18R6KT-mini-Tn7-Km	8x10	
C2	KmR (5' link to amyDown_end, 3' link to puc18-mcs)	pUC18R6KT-mini-Tn7-Km	12x14	
D1	AmyDOWN (3' link to puc18)	Xcc strain IPO3076	9x13	993
D2	AmyDOWN -	Xcc strain IPO3076	9x11	968

Table 1C *Resulting plasmids from 1-step Gibson assembly.*

Resulting plasmid constructs:	Promoter used to drive GFP expression	Integrated resistance market at double cross over?
pl_A-B1-C1-D1_pl	PsarA-sGFP	With Km marker integration
pl_A-B2-D2-C2_pl	PsarA-sGFP	Can be markerless
pl_A*-B1*-C1-D1_pl	Pamy-sGFP	With Km marker integration
pl_A*-B2*-D2-C2_pl	Pamy-sGFP	Can be markerless

The final consensus region was annotated in Geneious. For strain Xcc4050 (IPO3067_Pamy-GFP) a similar approach was taken, but this time the library was made of a genomic DNA of a single strain. The flow cell used for the previous library prep was washed according to the manufacturer's instructions, and loaded again. A total of 43.083 reads were obtained, and reads were binned using BLAST based on the presence of the sGFP coding sequence/ All 113 reads containing sGFP were trimmed for length and a consensus sequence was calculated using Canu as described above.

Screening for fluorescence

Colonies from Xcc transformations were screened for GFP fluorescence using a Biorad Chemidoc MP imager (filterset Epi-blue, 460−490 nm excitation; 518–546 nm emission) to detect GFP. If not obvious from the Biorad screening due to low signal to noise ratios, a colony was suspended to OD_{600} of 1.0 in NB medium and a spectral scan was performed for both the excitation and emission spectra (ex. scan 290-530, em 560; ex. 450nm, scan 470-580nm) to detect a specific sGFP signal using a BMG labtech Clariostar plate reader equipped with dual monochromators.

Effect of starch on sGFP production

The study the effect of the addition of starch to the growth medium on sGFP expression, strains were cultured overnight in ½ strength TSB from a -80°C stock. The next morning, the culture was diluted to an OD_{600} of 0.02 in ½ strength TSB, or ½ strength TSB with 0.01; 0.05, 0.1 and 1% starch (w/v) added. 150ul of the culture was transferred to each well of a transparent flat bottom 96 well plate (greiner), and samples were incubated at 30°C, shaking at 400rpm in a double orbital. For each condition 4 wells were used. Measurements of absorbance at 600nm and GFP fluorescence (ex. 488 bp 20, emission 520 bp 30) were taken every 5 minutes. Values were averaged over 4 wells, and corrected for background fluorescence of sterile medium with equal concentrations of starch and/or growing cultures without GFP present.

Virulence and stability of the mutants

The virulence of the mutants was tested on rapid cycling *Brassica oleracea* CrGC stock 3–1 (RCB) plants, which is an open pollinated variety. Plants were grown as described in Van der Wolf et al. (2000). Four weeks after sowing, two leaves per plant were rubbed using plastic gloves to remove part of the hydrophobic wax layer in order to enhance inoculation. Leaves were inoculated with a cotton swap dipped in a suspension of Xcc grown on TSA (+ appropriate antibiotics) with a density of approximately 10^7 cfu/ml (OD620=0.1) in Phosphate Buffer with 0.1% Tween20 (PBT). Plants were placed in a humid chamber for two days, after which they were grown at 24 °C and a RH of ca. 60%. Plants were visually observed for symptom development at 7, 14 and 21 dpi using the disease scores as described by Massomo et al. (2004). Mock-inoculated plants served as a control. Plant tissues were weighed and extracted in double the amount of PBT after which the (diluted) extracts were spread-plated on the semi-selective mFS medium.

Detection of GFP-tagged bacteria using various microscopical techniques

The GFP-tagged strains Xcc IPO4050 and IPO3555 were grown on YGM plates for three days at 27 °C and the fluorescence of the mutants compared using a epifluorescence stereomicroscope (ESM) (Leica MZ FL III, DE) equipped with a mercury high-pressure photo-optic lamp (Hg 50 W/AC) and GFP 2 filter cube. The fluorescence of Xcc IPO4050 was also evaluated in agar pour plates, for which bacterial suspensions were mixed (while shaking) with liquefied YGM medium, which was cooled down to a temperature of 50 °C. Pour plates were incubated for three days at 27 °C and the colonies were observed using ESM and also by bright field illumination. Young immature siliques of cauliflower plants (*Brassica oleracea* var. *botrytis*, nr 103598.1451 = cv. Balaka, RijkZwaan, De Lier, NL) were

inoculated at the basis of the siliques and the fluorescence determined at 14 dpi in transections made halfway the siliques. For confocal laser scanning microscopy (CLSM), a Leica DM5500Q microscope was used with an excitation wave length laser of 488 nm and a 505 nm filter for emitted light. We used 10x and 63x water immersion objectives and a 10x ocular. Pictures of fluorescent tissues were made with a Leica Digital System camera and of visible light with a Nikon digital microscope camera, both connected to the CLSM.

Leaf discs of the cauliflower plants with a diameter of 1 cm^2 were inoculated on the abaxial side with 20µl of a suspension of IPO4050, IPO3555 or with IPO3078 (parental wild type strain of IPO3555) using a density of approximately 10^{10}, 10^9, 10^8 or 10^7 cfu/ml. The suspensions were evenly distributed with a cotton wool slab on the discs damaged prior to inoculation using the tip of a pipette to remove partly the hydrophobic wax layer of the leaf surface.

Statistics

Numerical data were recorded and analysed in Microsoft Excel 2010, and when appropriate in the 18^{th} edition of the statistical program Genstat (VSN International, Rothamsted UK). In the pathogenicity test, comparison of areas under the black rot progress curves was made by General Analysis of Variance (ANOVA) and the Least Significant Difference (LSD, 5% level) of means was calculated.

Results

Identification of a suitable locus for chromosomal integration of sGFP gene

To facilitate the screening for correct clones during chromosomal integration, a double crossover should preferentially result in a phenotype that can be easily screened for in the laboratory. A locus used for this purpose in other bacteria (e.g. *Bacillus subtilis*) is the gene encoding a secreted amylase (Shimotsu & Henner, 1986). Secreted amylase activity can be easily visually screened for by growing the strains on agar plates containing starch. Ideally, replacing the amylase gene with a marker gene will result in loss of amylase activity. In the genome of Xcc strain ATCC 33913 three amylase genes are annotated: NP_638705; NP_636139 and NP_635528. The encoded proteins were analysed by SignalP 4.0 (Petersen et al., 2011), and only NP_636139 was annotated to contain a signal sequence suitable for secretion of the enzyme. Using TSA plates with 1% starch added, we confirmed that all our Xcc strains did secrete an enzyme with amylase activity, as clear halo's were visible surrounding the colonies (data not shown). Simultaneously, this confirmed the activity of the amylase promoter driving expression of this gene, and as such, using the native amylase promoter could be a good way to drive sGFP expression. We therefore designed primers to allow for integration of the GFP expression cassette at this locus.

Other considerations for selecting a suitable region for integration of a reporter gene would be the gene copy number. A locus near to origin of replication will be advantageous, as in rapidly growing cells there usually are more copies of this part of the chromosome present compared to loci close to the terminus (de Jong et al., 2017). We therefore singled out three other potential loci in the Xcc genome. Although we did not experimentally verify them, we expect these loci also to be of use in future studies, which is why we present them here as well (Table 2). The selection criteria for these loci are discussed in detail in (de Jong et al., 2017), and are shortly: near the origin of replication, in between two genes, downstream both of them (in order not to interfere with the promoter regions), presence of single restriction site in region facilitates ease of cloning downstream. Using the method as described in de Jong et al, 2017, integration at the selected loci will result in an addition of one of more genes, without replacing or removing any bases.

Table 2 *Locations on the Xcc chromosome suitable for marker gene integration.*

Location on chromosome	Upstream gene	Non-coding region	Site available in sequence	Function
12.9 kb	ExbD2-stop=12.959-14.113 stop-pdxJ (compl.)	= >1000 bp for integration	BstBI (XbaI also available)	Gene for ferric iron uptake (non-essential) (Wiggerich & Pühler, 2000)
23.1 kb	serA-stop=23.101 – 24.036 stop-ctp (compl.)	= ~1000 bp for integration	ClaI-site (DAM)	D-3-phosphoglycerate dehydrogenase
36.4 kb	yahK-stop=36.470 – 37.451 stop- Xcc0030 (compl.)	= ~1000 bp for integration	KpnI site (XbaI also available)	Alcohol dehydrogenase

Ease of transformation, antibiotic resistance and amylase activity of Xcc strains

Several Xcc strains from our culture collection were analysed for genetic transformability, antibiotic resistance and amylase activity. Based on this analysis strains IPO3076 and IPO3367 were selected for further studies, and the Kanamycin resistance cassette from plasmid pUC18R6KT-mini-Tn7-Km (Addgene plasmid #64969, (Choi et al., 2005)) was selected as a selection marker.

Resistance profiles

Many of the strains of Xcc in our collection did show high intrinsic resistance against commonly used antibiotics. We tested ampicillin (100µg/ml), kanamycin 25-100µg/ml, chloramphenicol 10µg/ml and erythromycin (100µg/ml). High background resistance showed to be a problem for many strains. After plating these strains, there were many colonies appearing on plates containing the antibiotic concentrations mentioned above. Specially ampicillin and chloramphenicol did not work well for selection. Kanamycin showed much lower background resistance, and was also successfully used on plasmid pUFZ75 (Zhang et al., 2009). We therefore decided to use a kanamycin resistance gene as a marker to select for chromosomal integration.

Construction of integration vectors

To assemble the integration vector, the empty backbone was cut and all PCR amplified fragments were assembled in a 5 point Gibson assembly using NEB HI-FI kit, followed by direct transformation to an *E.coli* PIR strain. We did not have an *E. coli* PIR strain without vector present in our strains collection, so we used the strain containing the empty vector as host. We then selected for the Kanamycin resistance marker only present on the new plasmid, assuming that two plasmids with identical origins of replication would be unlikely to co-exist. This indeed was the case, we obtained many good, bright green clones. Restriction analysis proved that none of these colonies contained more than 1 variety of the plasmid (not shown). *E.coli* clones containing constructs with the SarA-P1 promoter driving sGFP expression were bright green in normal daylight (Figure 1). In contrast, *E. coli* containing plasmids containing the native Xcc amylase promoter were hardly fluorescent, also not when using correct excitation and emission filters in the Biorad imager.

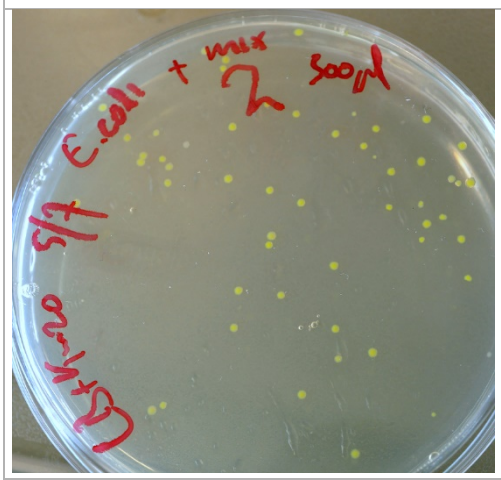
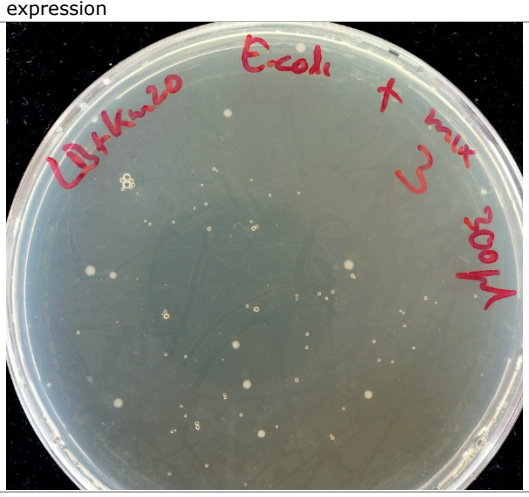
E. coli colonies after transformation of ligation mixture #2, containing the SarA-P1 promotor driving GFP expression	*E.coli* colonies after transformation of ligation mixture #3, containing the Xcc native amylase promoter driving GFP expression
	
pl_A*-B1*-C1-D1_pl AmyUP_P-RBS (5' link to puc18) - RBS-sGFP (5' link to AmyUP and 3' link to KmR) - KmR (3' link to Amydown_start) - AmyDOWN (3' link to puc18)	pl_A-B1-C1-D1_pl AmyUP_NoP (5' link to puc18) - PsarA-RBS-sGFP (5' link to AmyUP and 3' link to KmR) - KmR (3' link to Amydown_start) - AmyDOWN (3' link to puc18)

Figure 1 *GFP expression visible in daylight conditions for two of the constructed plasmids, when present in E.coli.*

Integration into Xcc genomes

To enable successful targeted integration in the Xcc chromosome, the constructed integration vector contains two Xcc genome fragments flanking the amylase gene. These fragments are both approximately 1000bp in length. Clones where the plasmids had integrated successfully could be selected by screening for fluorescent colonies. Of these fluorescent colonies, successful double cross over events could be detected by plating on plates containing starch. The absence of a halo indicated the loss of amylase activity. Two constructs were made. The first one was designed to replace the amylase gene from start to stop codon with an sGFP construct which expression was controlled by the SarAP1-promoter originally from *S. aureus* (Pang et al., 2010). This promoter was shown the be very active in a range of bacteria next to *S. aureus*, such as *E. coli* or several *Bacillus* species (de Jong et al., 2017). However, in *Xanthomonas* this promoter did not drive strong expression, and only low fluorescence signal could be detected above background (Figure 2, plate reader data). In contrast, the second construct was designed such that the sGFP expression was controlled by the native promoter of the amylase gene, which resulted in a level of GFP expression that was readily detected and much higher than the background fluorescence signal.

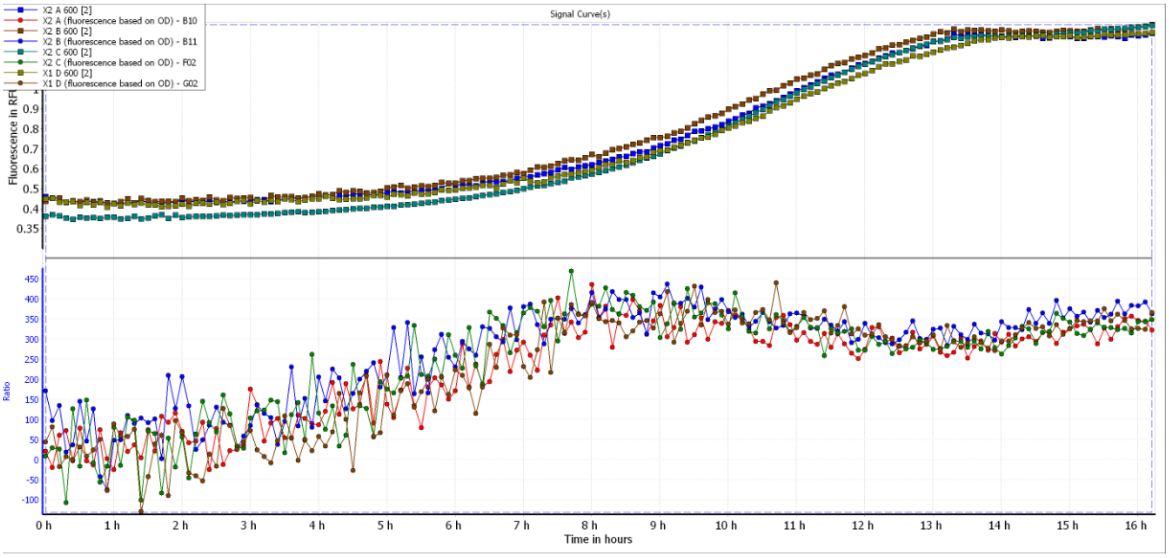


Figure 2 *expression of GFP in Xcc strain 3076, corrected for background fluorescence. A weak but detectable expression of GFP can be detected, this is not influenced by the concentrations of starch added to the growth medium.*

For both constructs, efficiency of transformation was low, and about 10-25 fold lower compared to transformation with a control replicating plasmid such as pUFZ75-GFP (data not shown). Also, often background colonies would appear rapidly. Nevertheless, positive colonies could be selected aided by the visual screening for GFP expression using the Biorad imager.

Interestingly, a successful double cross over event occurred readily with the PsarAP1-sGFP_KmR construct, resulting in the absence of amylase activity on plate. With the construct containing the native amylase promoter, we did not pick up successful double cross over events, and instead obtained strains with good GFP expression which also still showed amylase activity, indicating an intact amylase gene, and potentially only a single-crossover event.

Whole genome sequencing to verify chromosomal integration

To verify in detail how the plasmids had integrated in the Xcc genome we performed whole genome sequencing using the Oxford Nanopore MinION. This indicated successful double cross over for the sarAP1-plasmid_sGFP (Figure 3-2). For strain IPO4050 (3367-PamyGFP) the plasmid was integrated by single cross over and copied 3 times into the genome (Figure 3-3). For strain IPO4048 (IPO3076-Pamy-GFP) the plasmid was integrated by single cross over fully once Figure 3-1).

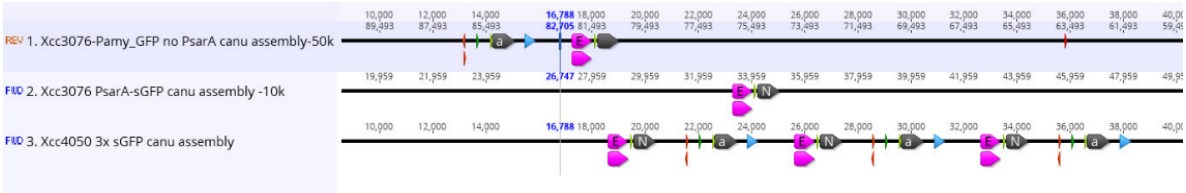


Figure 3 *Overview of genomic position and copy number of the integrated plasmid. Observations: Downstream amylase gene sequence not identical to what is present in the database, so more errors observed. Fig. 3-1. Xcc3076-Pamy-GFP = Xcc4048: single cross over at amy locus, full plasmid integrated and left behind intact amy-gene Fig. 3-2. Xcc3076-PsarA-GFP = Xcc4047: correct double cross over. Fig. 3-3 Xcc3367-PamyGFP = Xcc4050: single cross over with plasmid campble-ed in the genome tree times. Left intact amylase gene behind. No halo on plate.*

sGFP expression *in vitro* with/without starch

Initial observations on 1% starch plates showed stronger GFP fluorescence of strains Xcc4050 and Xcc4048 compared to plates without starch, indicating that the amylase promoter directing sGFP expression was upregulated in conditions with abundant starch. We set out to test this using varying starch concentration. Strains were grown in 96 well plate in a plate reader. The wildtype strain (IPO3076) and strain expression sGFP constitively from the SarAP1- promoter (IPO4047) were used as a controls to be able to calculate sGFP expression timing and strength during growth. As can be seen in fig. 4, optimal expression of sGFP was observed when 0.1% of starch was added to the growth medium. Higher starch concentrations caused a reduced sGFP expression.

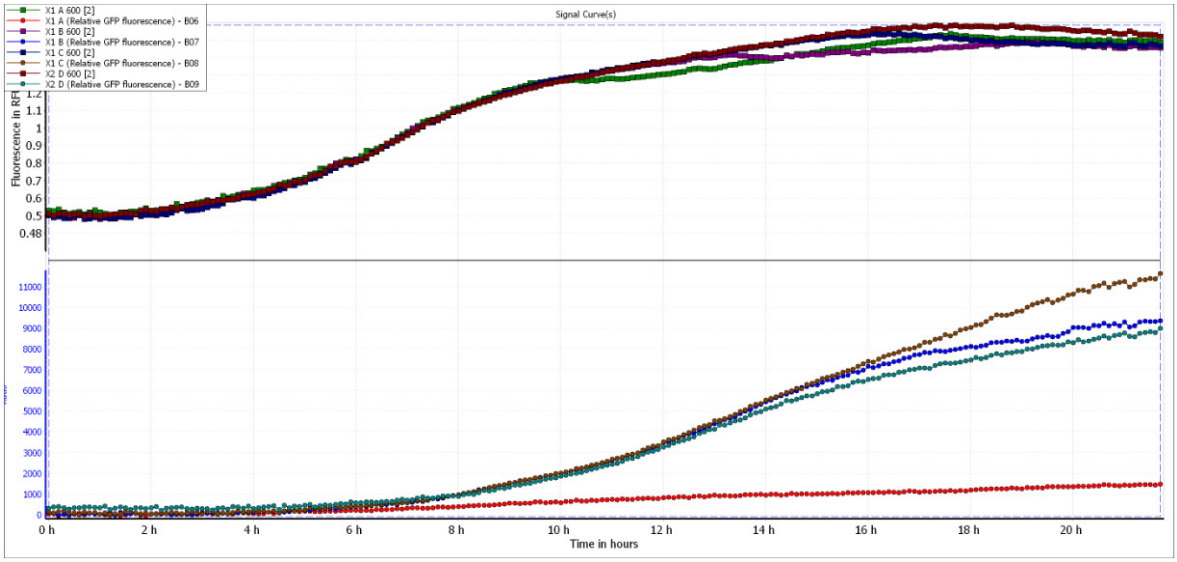


Figure 4 *Growth curve (OD600) and fluorescence signal of strain IPO4047 when grown with different concentrations of starch in the growth media. B6= no starch added, B7=0.05%, B8=0.1%, B9=1% starch added. The expression of GFP is optimal at a starch concentration of 0.1%.*

Strain 4050 displayed a bright fluorescence on YPG plates similar as IPO3555 using ESM (Figure 5A and B). The GFP signal was also observed with ESM and even weakly with bright field illumination if the bacteria grew in so-called pour plates (Figure 5C and D).

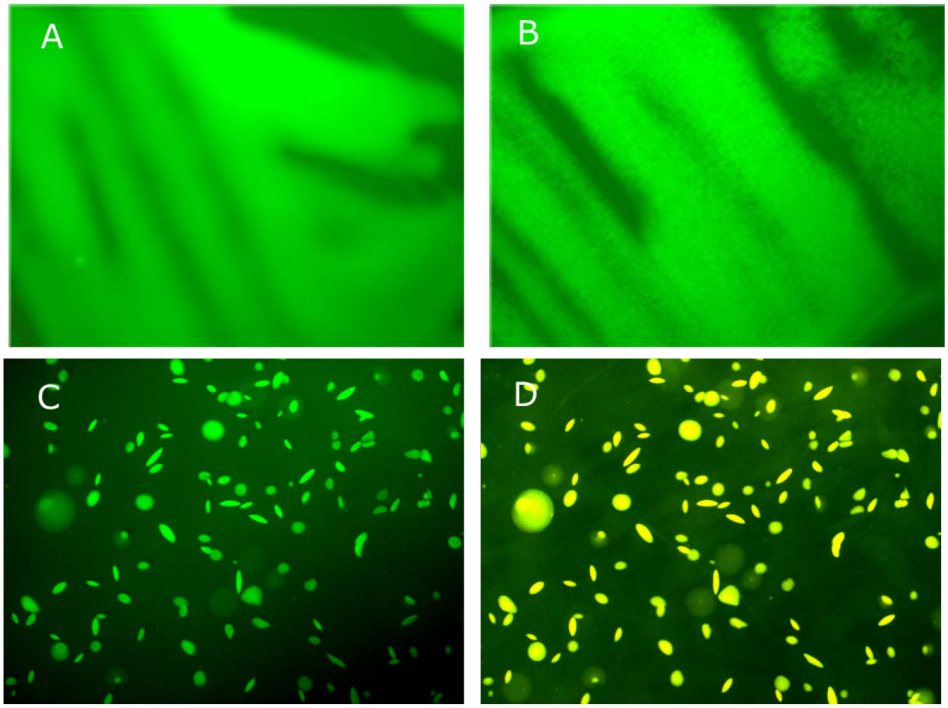


Figure 5 *A-C. Epifluorescence stereo microscopy images of the GFP tagged strain of Xanthomonas campestris pv. campestris IPO4050 (A), of IPO 3055 (B) grown on YPG medium and of strain IPO4050 grown in pour plates of YPG (C). In image D, strain IPO4050 growing in pour plates of YPG is visualized by bright field microscopy.*

Virulence in Rapid Cycling *Brassica oleracea* plants

The virulence was determined by leaf inoculation of Rapid Cycling *Brassica oleracea* plants. The AUDPC values of the various mutants (IPO 4047, IPO 4048, IPO 4049, IPO 4050 and IPO 3555) were not significantly different (P=0.05) from those of two wild type strains (IPO 3076, IPO 3367), indicating that the virulence of the strains was comparable (Table 3).

Table 3 *Virulence of GFP-tagged strains of Xanthomonas campestris pv. campestris in rapid cycling Brassica oleracea Crdc 3-1.*

inoculum	WT parental strain	GFP-construct	ID, origin	AUDPC[a]	
water	-			14	a
IPO3076	-	-	NCPPB528, UK	81	b
IPO4047	IPO3076	P3-2	-	94	bcd
IPO4048	IPO3076	P3-2	-	69	b
IPO3367	-		VN1, Russia	85	bc
IPO4049	IPO3367	P3-2	-	85	bc
IPO4050	IPO3367	P3-2	-	81	b
IPO3555	IPO3078	PUFZ75-GFP	PHW824-1, 1986, USA,	78	b

[a] AUDPC = area under disease progress curve was determined three weeks after leaf inoculations.

Values followed by different letters are not significant different (P=0.05).

Mock-inoculated plants did not show any typical symptoms. In one of the stems of the mock-inoculated plants (out of five), fluorescent colonies were observed with a typical Xcc appearance, likely due to cross-contamination. The mutants were able to migrate from the inoculated leaf blades via the petioles into the stems, although IPO3555 seem to have a reduced ability for systemic movement. For IPO 4049 and 4050 the stability of the GFP-expression in the mutants was high; all colonies on plates were fluorescent. However, for IPO4047, IPO4048 and in particular for IPO3555, part of the cells in the population had lost its ability to express GFP (Table 4).

Table 4 *The ability of (GFP-tagged) Xanthomonas campestris pv. campestris (Xcc) strains to migrate in rapid cycling Brassica oleracea plants from leaves to petioles and stems, and the stability of the GFP expression in planta.*

Inoculum	WT parent	Migration to		stability GFP-expression
		petiole	Stem	
water	Na	0/5[1]	1/5[2]	na
IPO3076	Na	4/5	5/5	na
IPO4047	IPO3076	5/5	4/5	reduced
IPO4048	IPO3076	5/5	3/5	reduced
IPO3367	Na	5/5	4/5	na
IPO4049	IPO3367	5/5	4/5	high
IPO4050	IPO3367	5/5	4/5	high
IPO3555	IPO3078	5/5	2/5	low

[1] Number of petioles positive for Xcc/total number tested by dilution plating on the semi-selective medium mFS.

[2] Typical green fluorescent colonies observed, likely due to cross-contamination.

[3] Colonies with a typical appearance for Xcc were observed for by epifluorescence stereomicroscopy.

Na = not applicable.

sGFP expression in in vitro and in planta

Using CLSM, bright green fluorescent cells were observed in parenchymatic and vascular tissues of valves, replum and septa of siliques inoculated with IPO4050 (Figure 6). The fluorescence was similar to IPO3555, whereas in mock-inoculated no fluorescent cells were found (results not shown).

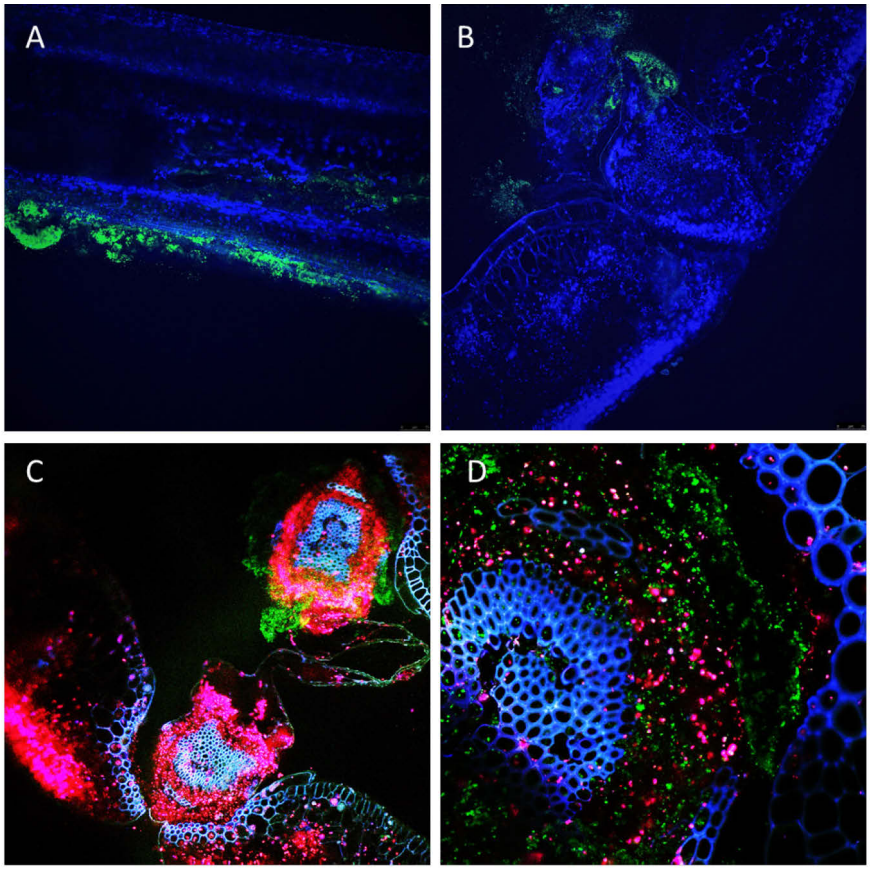
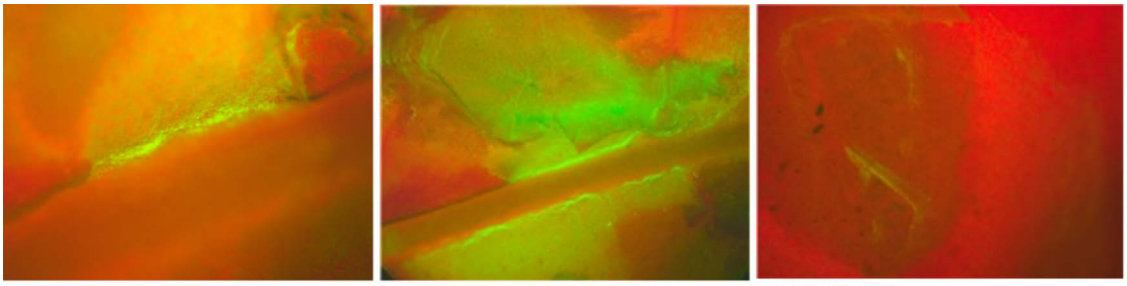


Figure 6 *Confocal lase scanning microscopy images of siliques inoculated at the basis with Xanthomonas campestris pv. Campestris IPO4050 and incubated on a blotter wetted with a Scc semi-selective medium for 3 days. A. Green fluorescent signal is predominantly on the externa surface of the siliques. B. Colonization of tissue near to septum and repium. C. Colonization of septum and replum. D. Detail showing abundant colonization of parenchyma cells of the mesocarp near to lateral vascular bundles.*



IPO4050, 10^{10} cfu/ml IPO3555, 10^{10} cfu/ml IPO3555, 10^{9} cfu/ml

	Density (cfu/ml)				
Treatment	10^{10}	10^{9}	10^{8}	10^{7}	0
IPO4050	+	-	-	-	-
IPO3555	+	+	-	-	-
IPO3078	-	-	-	-	-

Figure 7 *Sensitivity of epifluorescence stereomicroscopy for monitoring GFP-tagged strains of Xanthomonas campestris pv. campestris on Brassica oleracea leaves. 20 µl of the suspensions was deposited on leaf discs with a diameter of 1 cm^2.*

If suspensions were deposited on leaf discs of cauliflower plants, for strain IPO4050, a fluorescence signal was only found at a density of 10^{10}, 10^{9}, 10^{8} or 10^{7} cfu/ml whereas for IPO3555, both at 10^{10}, 10^{9} cfu/ml (Figure 7). It indicates that in general high densities are required to allow monitoring of GFP-tagged strains, but IPO3555 exhibits a stronger fluorescence than IPO4050 which is confirmed by other observations.

Conclusion and Discussion

A vector was successfully constructed for chromosomal integration of reporter genes via both single and double cross over at the locus of the native secreted alpha-amylase genes located on the chromosome of Xcc. Using the amylase gene for integration is a technique used a lot in model organisms such as *Bacillus subtilis* and was also found suitable for Xcc as only one copy is present. Interestingly, whereas the *S. aureus* SarA-P1 promoter was strongly active in *E. coli* host, very low expression of this construct in Xcc was found. In contrast, the native amylase promoter did not drive expression of GFP in the *E.coli* cloning host but had much stronger activity in Xcc compared to the non-native *S. aureus* SarA-P1. This is of interest, as the SarA-P1 promoter is active in a wide range of hosts, such as *Staphylococcus aureus*, *Bacillus subtilis*, *Paenibacillus* and *E. coli* (de Jong et al., 2017). Not surprisingly, the most bright fluorescent colonies selected were those with multiple integrated copies.

Strains in which GFP is expressed on a replicating plasmid are instable and bacterial cells readily losses the ability to express GFP during multiplication. In contrast, a stable expression of sGFP was found from the alpha-amylase promoter during at least five generations, even if selection pressure from antibiotics was absent. In addition, none of the Xcc bacteria with the chromosomal integrated sGFP had lost its fluorescence when isolated from plants at several weeks after inoculation (results not shown).

Using this newly engineered strain we were able to study its distribution in cabbage, showing colonization of the xylem and the leaves comparable with the wild type strain. We also showed that the strain with the single cross-over (IPO4050) displayed a high fluorescence in confocal laser scanning microscopy. However, in epifluorescence stereomicroscopy, the fluorescence intensity of IPO4050 was lower than that of a strain transformed with GFP expressed on a replicating plasmid.

The use of long read nanopore sequencing using the MinION sequencer enabled rapid and complete analysis of the way the plasmids were integrated in the genome and allowed us to explain the observed phenotypes. Long read nanopore sequencing supported a rapid check of integration. It was found that a single cross over leaves an intact copy of the gene. In this case integration could not be verified by the lack of amylase activity, but the presence of GFP fluorescence made it possible to screen for integration without PCR. Disruption of the amylase gene could make Xcc less competitive in *in planta* experiments.

The approach used here is expected to be applicable also to other (marker) genes next to GFP or other fluorescent proteins. As shown in Table 2, multiple other locations in the Xcc genome suitable for integration were considered. These locations have not yet been tested, but might prove usefull aiming to integrate multiple different gene expression cassetess in a single strain.

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3 Translocation of *Xanthomonas campestris* pv. *campestris* in *Brassica oleracea*

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Abstract
Translocation of *Xanthomonas campestris* pv. *campestris* (Xcc) in *Brassica oleracea* var. *botrytis* vernalization-independent cauliflower plants was studied to investigate the pathways of the pathogen via infected leaves into the seed. After inoculation of petioles, spray-inoculation of young plants, inoculation of rachises or inoculation of pedicels, the colonization of plant tissues was examined using dilution-plating. Studies with pedicel-inoculated plants were conducted with green fluorescent protein (GFP)-tagged strains of Xcc to allow also localization of the pathogen with fluorescence microscopy techniques, i.e. epifluorescence stereomicroscopy (ESM), fluorescence microscopy (FM) and confocal laser scanning microscopy (CLSM). A strain (IPO4050) was used in which the GFP expressing gene was integrated in the amylase locus on the chromosome and a strain (IPO3555) harboring GFP on a replicating plasmid.

After inoculation of petioles, no translocation to the stems was found as a rapid senescence of the inoculated leaves was found, resulting in leaf fall. This was found in repeated experiments both with cultivar Balaka and cultivar Campos.

Spray-inoculation of young plants of both cultivars in the six-leaf stage caused a severe symptom development resulting in necrotic leaves and incidentally in the development of symptomatic, dark-colored vascular tissue of the main stem. Translocation of Xcc in the developing plants was found to the top of the peduncles; for cv. Balaka in 1 out of 12 and for cv. Campos 4 out of 12 plants. Xcc was not detected in all inflorescences of an infected plant indicating an irregular distribution of the pathogen in the peduncles. The pedicels of infected peduncles, however, were all negative, indicating that the translocation of Xcc in spray-inoculated plants was limited to the peduncles.

Three independently conducted experiments were done with inoculated rachises, one at WUR with the GFP-tagged Xcc strain 4050 using cv. Balaka and two at the seed companies Syngenta and ENZA with Xcc IPO3078 using cv. Campos. Infections resulted in brown stripes on the rachis at the Xcc inoculation site and/or black/brown lesions on siliques. In the experiment at WUR, ca. 30% of the siliques became symptomatic. Using a dilution plating assay, infections were found in the inoculated rachises, pedicels, siliques, septa and in the seed, both externally (seed washings) as internally (seed extracts after disinfection). Only rarely a fluorescent signal was found on siliques or seeds using epifluorescence stereomicroscopy (ESM).

At the experiment conducted at Syngenta at 42-45 dpi, 14 out of 20 pedicels near to the inoculation site of the rachises were infected. Densities generally were high, 9 pedicels contained $>10^5$ cfu/pedicel, but unexpectedly only in 2 out of 20 siliques Xcc was detected. In a period between 28 and 42 dpi, seeds were collected. In total 50subsamples of 35 seeds were tested for the presence of Xcc. Wash water from the seed samples was analyzed. Thirty-two out of 50 subsamples were externally infected with densities ranging between 14 and 700,000 cfu per 35 seeds. We estimated that 2.9% of the seeds had external infections. Subsamples found externally infected were treated with hot water, crushed and the seed extract was tested to determine deep seated infections. From the 32 externally infected seeds, 15 samples were internally infected with densities ranging from 40 to

910,000 cfu per 35 seeds. We estimated that 1.6% of the seeds carried deep-seated infections. Xcc was not detected (externally) in 10 samples of 30 seeds of the PBS inoculated control plants.

In the experiment conducted at ENZA, 37-42 days after inoculation, 50% of the siliques were symptomatic, showing black tips, black/brown lesions, while some siliques were shriveled and exhibited a grey brownish color. In this experiment, pedicels and siliques from symptomatic siliques were sampled and symptomless pedicels and siliques from mock-inoculated plants. Xcc was detected in all 20 pedicels of Xcc-inoculated rachises with densities between 100 and 6000 cfu per ml of extract and in 19 out of 20 symptomatic siliques with densities of 100 to 170,000 cfu per ml of extract. In total 50 pools of 3-4 seeds (180 seeds in total) were tested from Xcc-inoculated rachises from which 15 pools were positive with densities ranging from 1 to uncountable cfu per ml of extract. In total 50 pools of 2 to 3 seeds (133 seeds in total) of mock-inoculated plants were analyzed. In all cases, extracts from mock-inoculated plant material were negative. In this experiment the total Xcc population density in extracted seed was determined. The disease prevalence of Xcc infected seeds was estimated at 9.5%

At 22 days after inoculation of the pedicels of cv. Campos with the GFP-tagged strains IPO3555 near to the proximal end of the siliques resulted in 100% infections of the basis of the silique and ca. 90% of the top of the silique for both of the GFP-tagged strains. In most cases high densities were found. Infections resulted in the development of black/brown lesions on siliques and/or in a necrotic tip. Siliques did not yield seed. It turned out that for cv. Campos, pollination should have been done manually; the use of bumble bees was not effective. Using FM and CLSM, bacteria were found mainly in vascular tissue and the parenchyma cells of the replum of the siliques. Incidentally, Xcc was located in the septum or found in biofilms attached to the endocarp.

In conclusion, we found that after early infections of petioles of individual leaves, transmission of Xcc into stems is limited as a result of leaf fall due to ageing and due to a hypersensitive response. Xcc, however could migrate from spray-inoculated leaves of young plants into the distal end of the peduncles. Although population densities in the peduncles could be high, no infections in the pedicels were found. Possibly, migration of Xcc into peduncles and/or the development of populations in peduncles was too late for migration into pedicels. If high densities of Xcc were inoculated in the rachises of peduncles, Xcc was able to migrate into the pedicel nearby the inoculation point and further into siliques and seeds with a high efficiency, even resulting in deeper seated seed infections. Inoculation of rachises resulted in severe symptom development in a high percentage of the siliques. Studies with fluorescence microscopy indicated that after pedicel inoculations Xcc migrated via the vascular bundle of the suture. Seeds may have become infected via vascular tissue in septum and funicle, but also via contact with Xcc growing as a biofilm on the endocarp of the valves of the siliques.

Introduction

Black rot, caused by the seed- and soil- borne bacterial pathogen *Xanthomonas campestris* pv. *campestris* (Xcc), is considered to be the most important bacterial disease of Brassica crops (Williams, 1980). Despite considerable efforts to produce Xcc-free seed, seed infections still occur (Williams, 1980, Gitaitis & Walcott, 2007). How seeds become infected is not fully known, but essentially there are two possible routes. The first is via infection of reproductive organs. If flowers or developing siliques become infected via airborne inoculum, it may result in infection and translocation of Xcc via vascular tissues of silique and funicle into the developing seed, or alternatively via contact of seed with infected tissues of valves or septa (van der Wolf et al., 2013, van der Wolf et al., 2019). Seed may also become infected after infections of roots or leaves (the second route). Root infections may result from soil-borne inoculum while leaf infection may result from air-borne inoculum carried by wind, aerosols, irrigation water, rain and pollinating insects, farm equipment and workers (Vicente & Holub, 2013). Xcc can move upwards in xylem sap and may reach the seeds via translocation through peduncles. However, scientific evidence is lacking.

This research was aimed to explore the potential risks for seed infections after leaf inoculation. Translocation of *Xanthomonas campestris* pv. *campestris* (Xcc) in vernalization-independent cauliflower plants was studied to investigate the pathways of the pathogen via infected leaves into the seed. Therefore the pathway was subdivided into trajectories. The migration of the pathogen was

studied after inoculation of petioles, after spray-inoculation of young plants, after inoculation of rachises or after inoculation of pedicels using dilution-plating. Studies with pedicel-inoculated plants were conducted with a green fluorescent protein (GFP)-tagged strains of Xcc to allow also localization of the pathogen with fluorescence microscopy techniques, i.e. epifluorescence stereomicroscopy (ESM), epifluorescence microscopy (EM) and confocal laser scanning microscopy (CLSM). A strain (IPO4050) was used in which the GFP expressing gene was integrated in the amylase locus on the chromosome and a strain (IPO3555) harboring GFP on a replicating plasmid.

Materials and methods

Bacteria and growth conditions
Xanthomonas campestris pv. *campestris* (Xcc) strains IPO3076 (NCPPB528, UK), IPO3555 (GFP-tagged strain of IPO3078 =PHW824-1, USA, based on replication of PUFZ75-GFP) and IPO4050 (GFP-tagged strain of IPO3367=N1, Russia, based on chromosomal integration of *gfp*) were used from the IPO bacterial (work) collection at WUR. Strains were stored for maximally one month at 17 °C on YDC plates, IPO3076 on YDC without amendments, IPO4050 on YDC amended with 20 µg/ml kanamycin monosulphate (Duchefa Biochemie) and IPO3555 on YDC with 50 µg/mL kanamycin monosulfate. For inoculation, bacteria were grown on TSA plates (Oxoid, CMO131), for IPO3555 and IPO4050 with the appropriate antibiotic concentration for 48 h at 25 °C. Isolation from plant tissues was done using mFS plates (Duchefa Biochemie) supplemented with 200 µg/ml cycloheximid. mFS plates were incubated for 5-7 days at 25 °C before counting pale green, mucoid colonies surrounded by a small zone of starch hydrolysis, typical for Xcc. In case of GFP-tagged strains, colonies were checked for fluorescence using epifluorescence microscopy (ESM) using a Leica MZ FLIII fluorescence stereomicroscope with filter GFP2. Suspensions were prepared in phosphate buffered saline (per liter: 8 g NaCl, 13.5 g $Na_2HPO_4.12H_2O$, 2 g $NaH_2PO_4.2H_2O$, pH7.2) or PBS supplemented with 0.01% Tween20 (PBST). The ratio of fluorescent and non-fluorescent colonies with a Xcc appearance showing starch hydrolysis was determined in all cases. For incubation of plant tissue on blotters, 1/10 strength of TBY (10 g L^{-1} tryptone (Oxoid), 5 g L^{-1} yeast extract (Oxoid), 5 g L^{-1} NaCl, pH 7.5) was used supplemented with 200 µg mL^{-1} cycloheximide, 3 µg/ml of D-methionine, 1 µg/ml of pyridoxine, 30 µg/ml of trimethoprim and 20 µg/ml Kanamycin (TBY^{ab}).

Inoculation of petioles (2016, 2017 and 2019)
Plant material. In 2016 and 2017, *Brassica oleracea* var. *botrytis* nr 103598.1451 = cv. Balaka, a vernalisation independent cauliflower breeding line of Rijk Zwaan was used. In 2016, seeds were sown in a seed tray with Horticoop seedling mix on 19 September and in 2017 on the 20th of September. Eight to ten days after sowing, seedlings were transplanted in 8 cm pots (Pöppelmann TEKU), after 24-34 days in 12 cm pots and after 56-59 days in 3L pots with Horticoop potting soil. Plants were grown till inoculation at 18/15 °C (day/night) and from inoculation onward at 24/20 °C (day/night), and a relative humidity of 55-60%. Plants were fertilized weekly. Sciarid flies were controlled by restricted watering of plants and trapping the insects with yellow sticky traps (Koppert). Grey mould (*Botrytis cinerea*) was also prevented by restricted watering and by two applications of chlorothalonil (Daconil, 0.3%) on the curds.

In 2016 and 2017, inoculations of petioles of cv. Balaka did not result in systemic infections of plants due to rapid ageing. This cultivar was also highly susceptible to fungal infections of cauliflowers, resulting in poor development of the inflorescences. Therefore, in 2019 two cultivars were used, i.e. cv. Balaka (provided by RijkZwaan) and cv. Campos (provided by Bejo). Plants were sown in two batches on 29 November 2018 and 20 December 2018 and grown at 24/20 °C (day/night temperature) and 50% RH as described previously till inoculation (at the six-leaf stage). Plants exhibiting fungal infections on cauliflowers were removed or (in case of light infections), fungal-infected cauliflower tissue was removed and curds treated with the fungicides Nimrod (Adama) or with Dacomil.

Petiole inoculation. One day before inoculation, plants were not watered; next watering was done shortly after inoculations. Two petioles per plants were inoculated with a suspension of Xcc IPO3076. In 2016 on 24 November (66 days after sowing) and in 2017 on 12 October at the transition to the leaf blade when curds were still closed but almost started to expand. In 2016, 20 mock-inoculated and

60 Xcc-inoculated plants were used. In 2017, 10 mock inoculated and 30 Xcc inoculated plants. A ten-times diluted suspension of Xcc in PBS was used with an OD620 of 0.1 (final density ca. 10^7 cfu/ml), using a syringe and a BD Microlance nr 3 needle (30G ½", 0.3 x 13mm), or petioles were mock-inoculated with PBS. In 2019, plants were inoculated on 17 January 2019 in the petiole (3 leaves per plant) as previously described. Plants were weekly observed for symptom development using the disease score table as described by (Massomo et al., 2004)

Disease scores
1 = no symptoms on the leaves
2 = minute necrotic lesions at the hydathodes to discrete vein-restricted marginal panels, no chlorosis beyond the margin of the lesions
3 = small marginal V-shaped lesion, 0.5 – 2 cm with narrow diffuse chlorotic margins
5 = small to medium V-shaped marginal lesion, vein blackening, lesions rarely progressing half-way to midrib
7 = expanding marginal lesions, prominent vein blackening, lesions frequently progressing half-way to midrib
9 = rapidly expanding marginal lesions frequently coalescing to give scorching and systemic invasion of the plant frequently accompanied with stunting and death of the plant

Sampling and analysis of petioles. In 2016, the entire petiole, ca. 2 cm of the lower stem and a composite sample of 5 cm of the basis of petioles was sampled, crushed, extracted in PBS (volume twice the weight of the plant tissue) and plated undiluted and 100 times diluted. In 2017, the basis (lower 5 cm) of all peduncles per plant were collected, weighed, crushed and mixed through a volume of PBS twice the weight of the plant tissue. Extracts were plated undiluted on mFS. In 2019, at 14, 22 and 29 dpi, fallen leaves from needle-inoculated plants were collected, while at 71 dpi, stems with the peduncles of individual plants were collected.

Spray-inoculation of leaves (2019)
Plant material. Two cultivars were used, i.e. cv. Balaka (provided by RijkZwaan) and cv. Campos (provided by Bejo). Plants were sown in two batches on 29 November 2018 and 20 December 2018 and grown at 24/20 °C (day/night temperature) and 50% RH as described previously till inoculation (at the six-leaf stage). Plants exhibiting fungal infections on cauliflowers were removed or (in case of light infections), fungal-infected cauliflower tissue was removed and curds treated with the fungicides Nimrod (Adama) or with Dacomil.

Inoculation. Plants were spray-inoculated on 18 January 2019. For this, one day before inoculation, plants were placed on plastic trays, covered with a plastic tent and the table was covered with water to increase humidity to enhance guttation. Plants were sprayed with a suspension of 10^8 cfu/ml of Xcc3078 in PBS or with PBS (mock-inoculated) using a Birchmeier Foxy Plus sprayer. During cauliflower formation, the humidity was reduced to 40% using a dehumidifier to reduce risks for fungal infections.

Sampling and analysis. At 11 dpi the first spray-inoculated plants were sampled (those exhibiting fungal infections). From each plant, four leaves with symptoms were taken, and individually stored in zip-lock bags. At 68 dpi, per plant the peduncle was divided in four equal parts, such that each part contained roughly the same weight of inflorescences. Plant material was stored in a bigger zip-lock bag at -20 oC prior to analysis. The upper 20 cm of the branched top of the inflorescences was collected (four subsamples per plant), surface sterilized with 70% ethanol, dried in a laminar flow cabinet, extracted in PBST and analyzed by dilution-plating on mFS medium as described previously. From subsamples of inflorescences positive in the dilution-plating assay, the pedicels were collected (cut with a scissors) and extracted as composite sample in 1 ml of PBST and analyzed by dilution plating.

Inoculation of rachises (2017/2018, 2019/2020)
Plant material. In 2017/2018, seeds of *Brassica oleracea* var. *botrytis* (nr 103598.1451 = cv. Balaka) were sown on 20 September and grown as described before.

Inoculation of rachis. One day before inoculation, plants were not watered; next watering was done shortly after inoculations. In the period from 4 January 2018 till 30 January, plants were inoculated in the rachis near to the pedicels of sections with overblown flowers and young siliques with strain 4050 in total eight times. A ten-times diluted suspension of Xcc in PBS was used with an OD620 of 0.1 (final density ca. 10^7 cfu/ml), using a syringe and a BD Microlance nr 3 needle (30G ½", 0.3 x 13mm), or petioles were mock-inoculated with PBS. On the 21st of January the first sampling was done. Only fluorescent (GFP-expressing) colonies with a typical appearance for Xcc were found on plates. The experiment lasted till 3 April 2018.

Symptom development. Siliques on inoculated rachises were checked weekly for symptom development.

Sampling and analysis of pedicels, siliques. At 10-14 dpi, the inoculation success was determined using ESM and by dilution plating on mFS. Symptomatic and asymptomatic siliques were inspected for a green fluorescence using ESM. Prior to plating, siliques were disinfected with 70% ethanol and their pedicels were aseptically cut off. Plant tissue was crushed in a 60 × 80 × 0.09 mm plastic bag, mixed through PBST (for siliques twice the weight of the plant tissue was used and for pedicels a fixed volume of 250 µl), and incubated for 10-15 min at room temperature. At 35-40 dpi, additionally green siliques with immature seeds were analyzed. They were aseptically split lengthwise with a scalpel along the septum and the valves were separated from the septa. Silique parts were transferred to a 120x120x2 mm black frosted plastic sheet in a square petri dish and inspected for green fluorescence using ESM. Interesting parts were cut out for analysis using CLSM. Pedicels were cut off from the septa and the various plant parts were analyzed by dilution-plating as described before.

Enhancement of the signal in ESM. Flowering cauliflower plants were inoculated in the main vein of the leaf, the stem and the rachises with 10^7 cfu/ml of IPO3555 (plasmid based fluorescence) and IPO4050 (gfp integrated in the genome) or plants were mock-inoculated (N=3). Transections adjacent to the inoculation point were incubated on a sterile blotter paper with TBY (10x diluted + antibiotics) with or without starch for 3 days at 25 °C. The tissues were observed for a green (GFP) fluorescence using ESM.

Sampling and analysis of seeds. Seeds of ripe siliques were harvested twice a week before they opened to avoid seed losses and stored in paper bags at a dry place until use. Siliques were gently crushed and impurities were manually removed before weighing the seeds. To assess external contamination with Xcc, seeds were transferred to Eppendorf tube 1 ml or 2 ml of cold PBST (2-4 °C) was added. Tubes were shaken at 700 rpm for 10 min on a Sarstedt orbital tissue plate shaker and the wash fluid was dilution plated on mFS medum. At 60-80 dpi, seeds were analyzed per silique as composite sample after incubation on blotters for 48-72 h Seed washings were analyzed and seed extracts after disinfection of seeds with ethanol (deep-seated infections).

*Confocal Laser Scanning Microscopy (*CLSM). For CLSM, a Leica DM5500Q microscope was used with an excitation wave length laser of 488 nm and a 505 nm filter for emitted light. We used 10x, 63x and 100x water immersion objectives. Pictures of fluorescent tissues were made with a Leica Digital System camera and of visible light with a Nikon digital microscope camera, both connected to the CLSM.

Direct analysis of siliques by CLSM. Silique tissues were cut by hand. Seeds were manually cut in three approximately equal thick parts from which the central part was used. Tissues were embedded in 0.2% molted multipurpose agarose (Roche, Ch) of 50 °C on a microscope slide.

Enrichment of Xcc in septa preparatory to CLSM. For each inoculation session three symptomatic siliques were sampled or if symptomatic siliques were not found, three asymptomatic siliques. For this, each silique was put into a petri dish and inspected for the presence of GFP-signal using ESM. Siliques, selected on the basis of the ESM results, were split aseptically lengthwise with a scalpel along the septum and the valves were separated from the septa. Septa were inspected by epifluorescence stereo microscopy (ESM) for the presence of a GFP-signal with a focus on funiculi and seed coats. GFP-positive plant parts were carefully cut out for analysis by CLSM. The septa negative for a GFP-

positive signal were transferred to a blotter wetted with TBY[ab] and incubated for three times 24 h at 25 oC. To study the effect of the supplement of starch, in some experiments 25 g/L of starch was added to TBY[ab]. After each 24 h period, septa were inspected using ESM and cut out in case GFP-positive parts were found, for analysis by CLSM. After CLSM, the tissue and seed pieces were processed within 24 h for plating on mFS to confirm the presence Xcc.

Enrichment of Xcc in seeds preparatory to CLSM. Twenty-five seeds from each of the 10 Xcc-inoculated and 25 seeds from each of the 3 mock-inoculated plants were collected. Seeds were incubated for 2x24 h on blotters in petri dishes wetted with 1/10 strength of TBY with antibiotics similar as described for septa.

In 2019/2020, experiments were conducted at Syngenta and ENZA using Xcc strain IPO3078 and cv. Campos. Reports are provided in Enclosure 1 and 2.

Pedicel inoculation (2019/2020)
Plant material. At 3 December 2019, 19 plants of cv. Campos, provided by RijkZwaan, in a stage just before flowering were placed in the glasshouse at 24/20 °C (day/night temperature). At 9 December a Minipol box (Koppert, NL) containing a colony of about 40 bumblebees (*Bombus terrestris* L.) was placed in the glasshouse for pollination. From 20 December till 7 January 2020, the temperature was decreased to 15 °C to slow down plant development and from 7 January onward the plants were grown at a constant temperature of 21 °C.

Inoculation. From 7 January till 23 January 2020, pedicels were inoculated with Xcc IPO4050 or with Xcc IPO3555. The distal end of the pedicels, near to the basis of the siliques was inoculated with a needle as previously described On the 29th of February siliques were scored for symptom development. For microscopy studies, siliques were sampled between 29 January and 19 February 2020.

Analysis by dilution-plating. Randomly sampled siliques were cut in two fragments of an equal length. The cross sections were analyzed individually. The weights of the fragments were determined, twice the weight of PBS was supplemented, the fragments were crushed and the extracts dilution-plated on mFS as described before.

Microscopy studies. From the proximal, middle or distal end of green immature siliques, transections were made cut by hand using a razor knife, and the sliced silique tissues were visually observed for the presence of a green fluorescent (GFP) signal (FM) or for green fluorescent cells (CLSM). ESM and CLSM was done as described before.

Results

Petiole inoculation (2016, 2017 and 2019)
In 2016, no typical black rot symptoms were found at 14 and 28 dpi after inoculation of petioles with Xcc IPO3076. At 28 dpi most inoculated leaves had died due to ageing and possibly due to a hypersensitive response. At 19 dpi, from a selection of 1 mock-inoculated plant and 5 Xcc-inoculated plants petioles, stems and peduncles were analyzed by dilution plating on mFS. The Xcc-inoculated petioles were all positive exposing high densities of Xcc, but stems and peduncles were all negative, indicating that no translocation of Xcc from petiole to stems had occurred.

In 2017, the best moment of scoring was at 28 dpi, but even then already 27% of the leaves had dropped from the plants. In two of the inoculated leaves black veins were observed. In none of the extracts of the peduncle basis from 30 sampled Xcc-inoculated plants and 10 mock-inoculated plants, Xcc was detected by dilution plating. Therefore the pedicels were not analyzed.

In 2019, after inoculation of petioles with Xcc IPO3076, the first chlorotic spots were found at 7 dpi. Symptoms developed into typical V-shaped chlorotic spots and black veins (Figure 1). As in former experiments, the inoculated leaves dropped within 14 days after inoculation due to ageing and

possibly due to a hypersensitive response. We assumed that systemic infections after leaf inoculations were unlikely and therefore plants were not further analyzed.

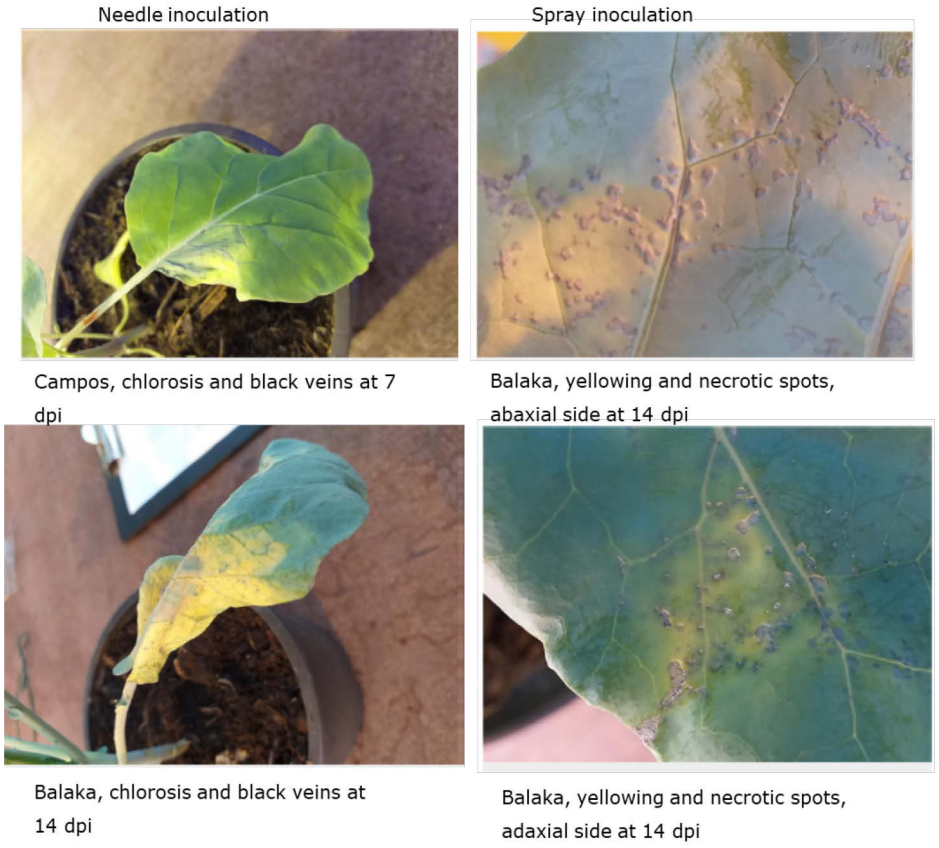

Figure 1 *Symptom development in cultivars Campos and Balaka after needle and spray-inoculation.*

Spray inoculation of leaves (2019)
Spray-inoculation of plants resulted initially (13 dpi) in the forming of small necrotic spots associated with the presence of stomata (Figure 1). In a later stage also typical black rot symptoms were found in both cultivars and at 68 dpi most leaves were largely necrotic as a result of severe symptom development. The Area Under the Disease Progression Curve (AUDPC) of both cultivars did not differ significantly ($P > 0.05$). Cultivar Campos was less prone to fungal infections than cultivar Balaka.

At 68 dpi, the rachises of each plant were separated in four equal parts (subsamples) and the upper 20 cm of florescence's of the subsamples was analyzed by dilution-plating. Cultivar Balaka yielded 2 subsamples of 1 plant (out of 12 plants) positive (Table 1). Cultivar Campos yielded 7 subsamples of 4 plants (out of 12 plants) positive. Mock-inoculated plants were all negative. All pedicels were collected and analyzed from plants with positive florescence's, but none of these 9 samples (2 Balaka subsamples, 7 Campos subsamples) were positive.

Table 1 *Infection incidence and rate of (subsamples of) peduncles of Brassica oleracea cultivars spray-inoculated with Xanthomonas campestris pv. campestris IPO3078.*

Cultivar	Treatment	Nr plants analysed[1]	Nr. plants positive	Nr. subsamples analysed[2]	Nr subsamples positive	Details[3]
Balaka	Water	3	0	12	0	0
	Xcc	12	1	36	2	Plant 1: 1xH,1xL
Campos	Water	3	0	12	0	
	Xcc	12	4	36	7	Plant 18: 2xH, 1xM
						Plant 11: 1xH
						Plant 12: 1xM
						Plant 16: 1xH,1xM

[1] The peduncles of the individual plant were analyzed by dilution plating on mFS.

[2] Per plant the peduncles was split in four parts with an equal weight of florescences, from which the upper 20 cm was analyzed.

[3] H = highly infected (>10.000 cfu/g), M = moderately infected (100 – 10000 cfu/g), L = low infection (100 cfu/g).

Rachis inoculation (2017/2018, 2020)

In 2017/2018, at 10-14 dpi, success of rachis inoculation was determined. All 25 Xcc-IPO4050 inoculated rachises that were analyzed yielded high densities of Xcc in the dilution-plating assay indicating that the inoculations were successful. Ten mock-inoculated rachises were all negative. From the Xcc-inoculated rachises, 20% (4 out of 20) of the symptomless siliques were positive and 30% (6 out of 20) of the corresponding pedicels, while from the 27 symptomatic siliques which were analyzed together with the pedicels, all samples were positive in the dilution plating assay. If samples were positive, in general high densities were present. Xcc was not detected in any of the 8 mock-inoculated pedicels or 8 siliques that were analyzed. All Xcc-typical colonies were fluorescent on mFS indicating that IPO4050 is expressing GFP in a stable way. However, IPO4050 did not or only weakly fluoresce on TSA. Incidentally, a typical green fluorescent spot was observed using ESM. In 3 out of 49 Xcc inoculated plants a GFP-signal was found at the border between green and symptomatic silique tissue. In the same siliques, a GFP-signal was also observed along the suture of the same siliques using the pathoscreen system (results not shown).

At 28 dpi, in total 3685 siliques from infected rachises present on 25 plants were inspected from which 971 (28% +/- 12.1%) showed symptoms. Symptoms were absent in siliques present on 10 mock-inoculated plants.

At 35-40 dpi siliques with and without symptoms were analyzed by ESM for the presence of a green fluorescent signal and with dilution plating for the presence of Xcc (Table 2). In the dilution plating assay, 33% of the pedicels, 17.9% of the silique base, 12.5% of the septum derived from siliques with an infected silique base, 12.5% and 28.6% of the seeds 28.6% were positive. Only rarely a positive signal was found in ESM (1 out of 9 pedicels). Samples from mock-inoculated samples were all negative.

Table 2 *Analysis of siliques, pedicels, septum and seeds at 35-40 days after inoculation of rachises of cauliflower plants with Xanthomonas campestris pv. campestris IPO4050 using epifluorescence stereomicroscopy (ESM) and dilution plating (without prior enrichment on blotters).*

		Plating			ESM		
Sample	Inoculum	N	Plating positive	% positive	N	ESM-positive	% positive[1]
siliques	Xcc	134	24	17,9	25	0	0
	Mock	27	0	0,0	5	0	0
Pedicels	Xcc	6	2	33,3	9	1	11,1
	Mock	1	0		1	0	
Septum	Xcc	8	1	12,5	9	0	0
	Mock	1	0		1	0	
Seeds	Xcc	7	2	28,6	5	0	0
	Mock	NT	NT	NT	NT	NT	NT

[1] 7 doubtful (possibly autofluorescence).

NT = not tested.

Table 3 *Analysis of silique-base, pedicels, septum and seeds at 35-40 days after inoculation of rachises of cauliflower plants with Xanthomonas campestris pv. campestris IPO4050 using epifluorescence stereomicroscopy and dilution plating (after enrichment on blotters wetted with a semi-selective broth).*

		Plating			ESM		
Sample	**Inoculum**	**N**	**Plating positive**	**% positive**	**N**	**ESM-Positive**	**% positive**
Silique base	Xcc	23	23	100,0	NT	NT	NT
	Mock	4	0	0,0	NT	NT	NT
Septum	Xcc	24	5	20,8	24	5	20,8
	Mock	5	0	0,0	3	0	0,0
Seeds (external)	Xcc	27	4	14,8	27	1	3,7
	Mock	3	0	0,0	3	0	0,0
Seeds (internal)	Xcc	27	6	22,2	NT	NT	NT
	Mock	3	0	0,0	NT	NT	NT

NT = not tested.

Additionally septa and seeds were analyzed from siliques found positive at the base after incubation on wetted blotters with YGM supplemented with antibiotics (enrichment) (Table 3). From the septa 20.8% of the samples were positive, from seed washings (external contaminations), 14.8% and from seed extracts (deep seated infections), 22.2%. In some cases, a typical GFP-signal was found on septa and also on the exterior of the seeds after incubation on blotters using ESM (results not shown). The silique and septum were incubated on a blotter and analyzed with CLSM. A clear signal was found on the external surface of the septum (Figure 2A) and on the tissue near to replum (Figure 2B).

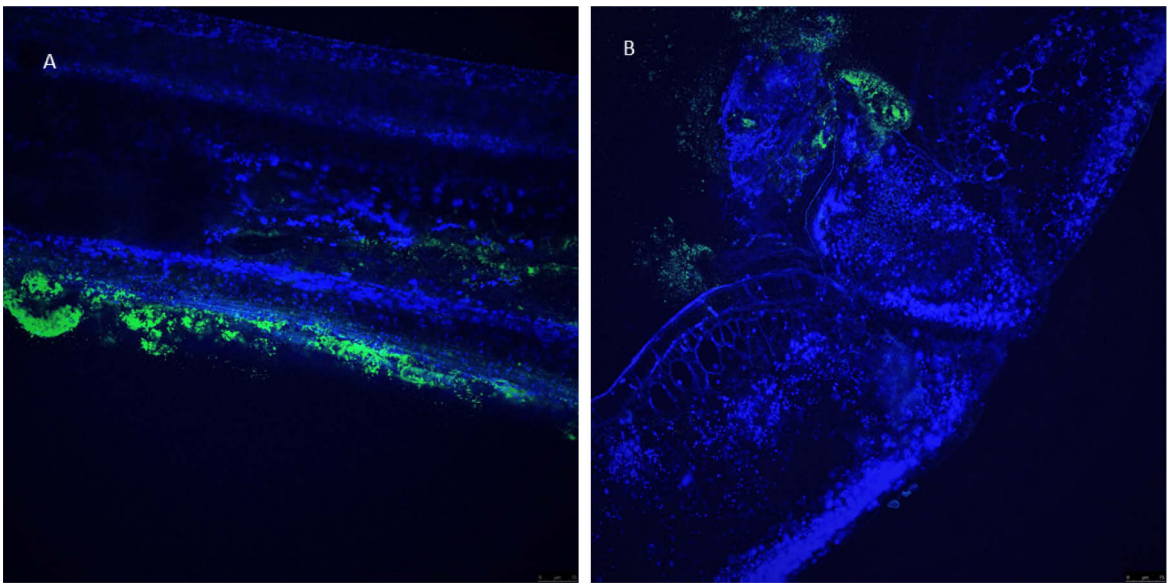

Figure 2 *CLSM image of a septum (A) and silique (B)from a plant inoculated with Xcc IPO4050 in the rachis and incubated on a blotter wetted with a semi-selective medium for 3 days. The signal is found predominantly on the externa surface of the siliques (A) (100x), but also at tissue near to the replum a fluorescent signal was found (B, 400 x).*

At 60-80 dpi, in total 114 seeds were analyzed from 21 half-ripe symptomless siliques, but infected with Xcc according to the dilution plating assay. Seeds were analyzed per silique as composite sample after incubation on blotters for 48-72 h (Table 4). Seed washings were analyzed (external infections) and seed extracts after disinfection of seeds with ethanol (deep-seated infections). Seed washings and seed extracts from 2 siliques were positive, one with 5 and one with 9 seeds. One seed showed a GFP-positive signal on blotters after incubation for 72 h. In total 20 seeds from 4 siliques harvested from mock-inoculated plants were negative.

Table 4 *Analysis of seeds at 60-80 days after inoculation of rachises of cauliflower plants with Xanthomonas campestris pv. campestris IPO4050 using epifluorescence stereomicroscopy and dilution plating (after enrichment on blotters) (results 2018).*

			ESM (individual seeds)		Plating (composites per silique)	
	N siliques	N seeds	GFP pos	GFP neg	Seed washings (external Xcc contamination)	Extracts of disinfected seeds deeper seated Xcc infections
mock inoc.	4	20	0	20	0	0
Xcc inoc.	21	114	1	113	2	4

Separate experiments were done to see if the GFP-signal in infected tissues could be further enhanced if starch was supplemented to TBY during enrichment. Incubation of plant tissues on blotters wetted with TBY resulted in a higher fluorescence in ESM for stem and rachis tissues inoculated with IPO3555 and for leaf and stem tissue inoculated with IPO4050 (Table 5).

In 2020, experiments were conducted at Syngenta and ENZA and the reports are provided in Enclosure 1 and 2.

Pedicel inoculations (2019/2020)
Plants (cv. Campos) inoculated with GFP-tagged Xcc IPO3555 in the distal end of the pedicels near to the basis of the siliques or mock-inoculated were analyzed in period between 23-35 dpi using EM and CLSM. Representative images are shown in Figures 3 and 4. A high percentage of the siliques became symptomatic. Siliques exposed dark brown or black irregular spots mainly at the distal end of the siliques (3A and 3B), not present in the mock-inoculated plants (3C). As symptoms progressed the distal end dried out and shriveled.

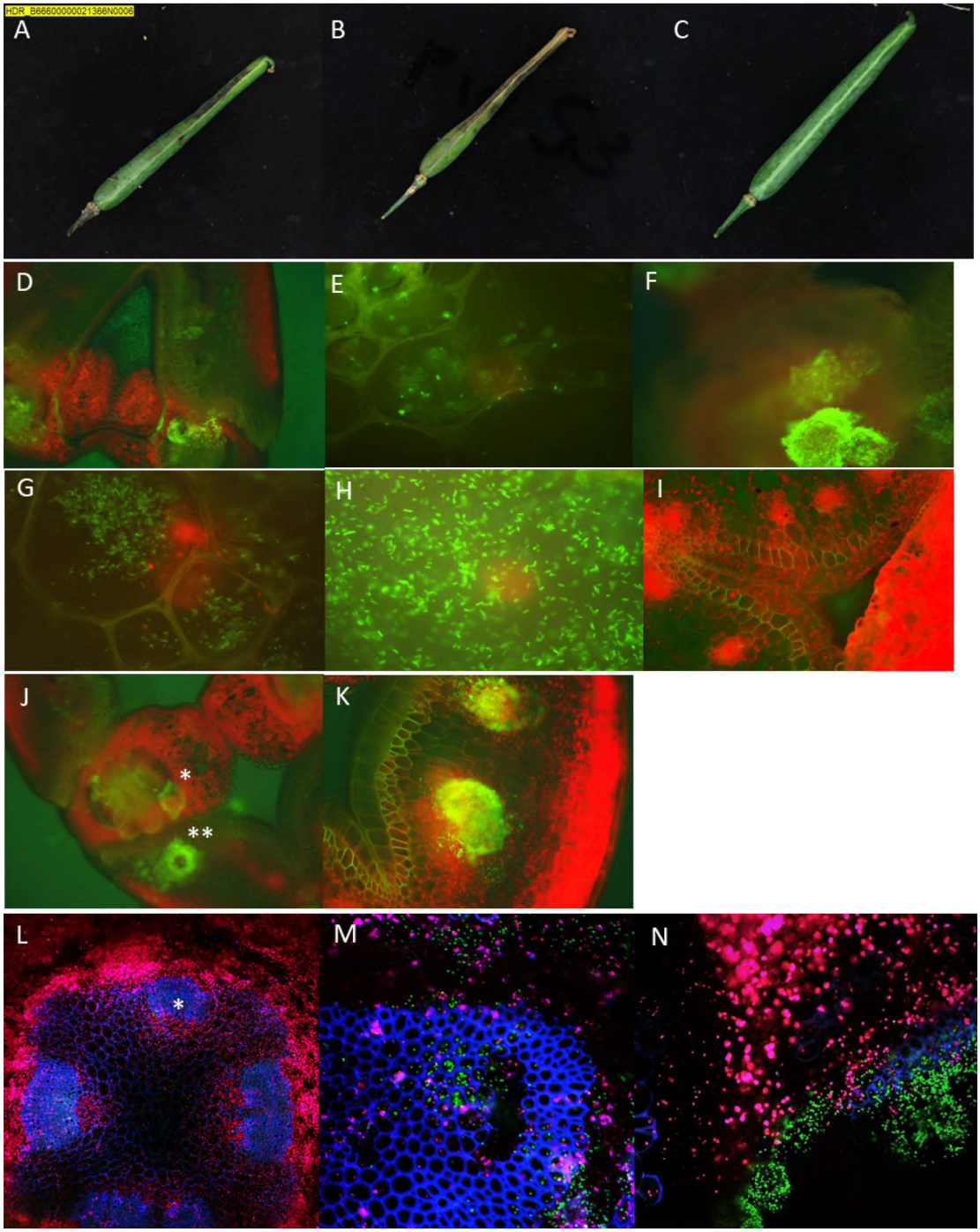

Figure 3 *Images of silique and silique tissues after inoculation of the distal end of the pedicels with Xanthomonas campestris pv. campestris (Xcc) IPO3555, near to the basis of the siliques at 3-5 weeks after inoculation. A-C. Bright field images of siliques A and B Xcc-inoculated, C Mock-inoculated. D-K. Epifluorescence Microscopy (EM) images of the silique base. D. Base with a fluorescent signal in the replum (20x), E. Fluorescent cells inside vascular tissue of replum (500x)). F. Leakage of a fluorescent biofilm from replum (250x). G. Fluorescent cells in lateral vascular bundles (500x). H. Fluorescent cells close to the endocarp (500x). I. Mock-inoculated. J. Fluorescent signal in vascular tissue of replum (*) and in a lateral vascular bundle (**) (50x). K. magnification of the lateral vascular bundle (250x). K-M. Confocal Laser Scanning Microscopy Images of the silique base. L. Fluorescent cells in lateral vascular bundles (100x). M. Fluorescent cells in xylem vessels of lateral bundles and surrounding parenchymatic cells (400x). N. Biofilms attached to the endocarp located near the replum (400x).*

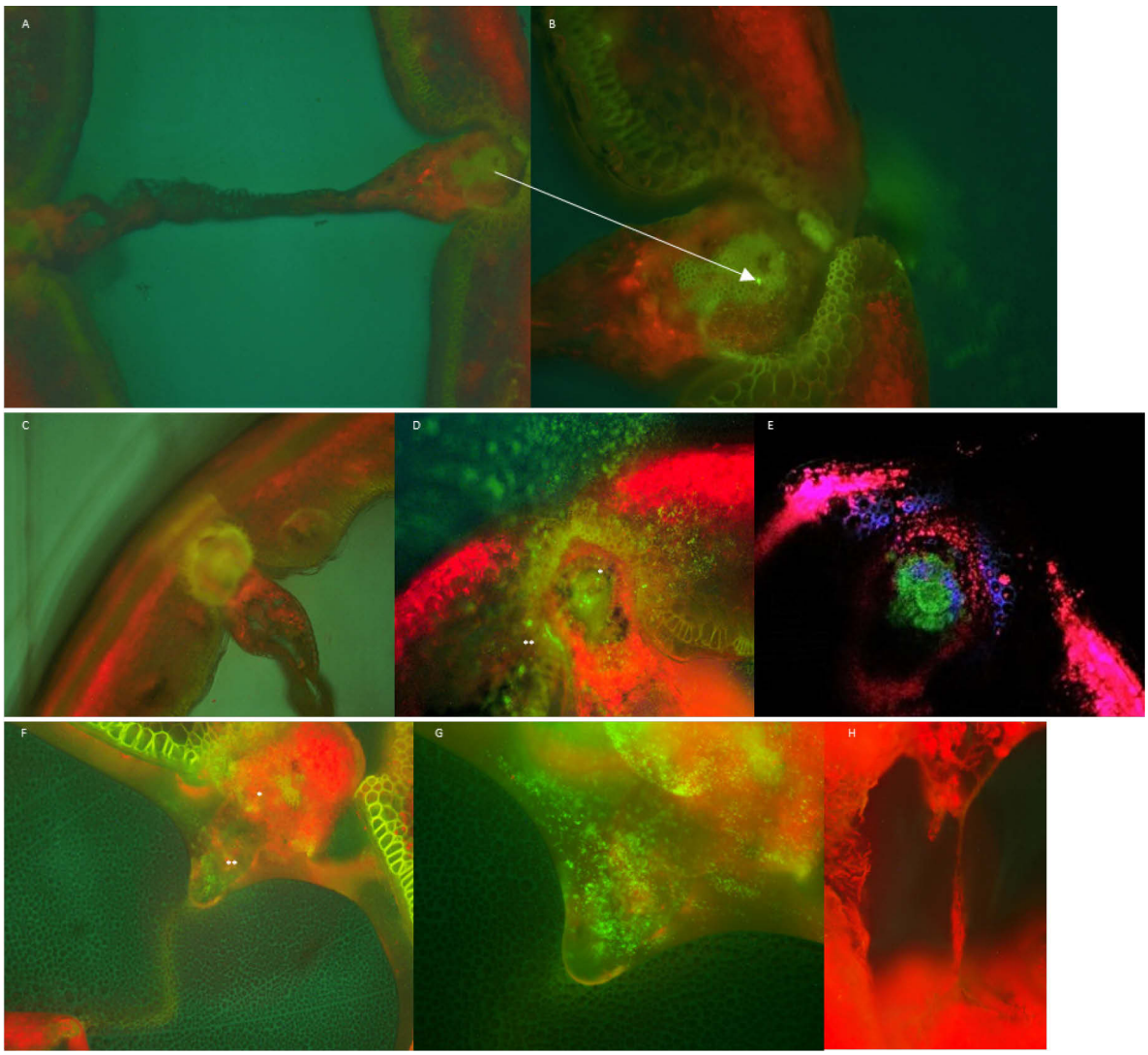

Figure 4 *Images of silique and silique tissues after inoculation of the distal end of the pedicels with Xanthomonas campestris pv. campestris (Xcc) IPO3555, near to the basis of the siliques at 3-5 weeks after inoculation. A-D. Epifluorescence Microscopy images of transections of the middle part of a silique, inoculated. A. Overview with septum and replum (100x). B. Magnification of a replum with a signal in vascular tissue (500x). C. Overview with septum and replum (100x). D. Fluorescent signal in replum (*) and endocarp tissue (**) (500x) E. Base with a fluorescent signal in the replum (400x). F-H. Epifluorescence Microscopy images of the top of the silique. F. Fluorescent signal in replum (*) and at the transition of replum to septum (**) (100x). G. Magnification showing fluorescent cells (500x). H. Mock-inoculated plants (100x).*

Plants did only produce a few seeds (<10) per plant and therefore the transmission to seed could not be studied. Instead, we analyzed the translocation through the siliques by analyzing the presence of Xcc in transections of the base, the middle and top of the siliques using EM and CLSM. Using EM, fluorescent spots were found in the replum at the base of the siliques (3D, 3J and 3K). At higher magnifications, fluorescent cells were found inside xylem vessels in the replum (3E). Incidentally a large cluster of fluorescent cells, likely a biofilm of Xcc cells was leaking out of the replum (3F). Fluorescent cells were also found in lateral vascular bundles (3G). Individual fluorescent cells were visible in the xylem vessels of lateral vascular bundles at higher magnification (3H). No typical green fluorescent (GFP) signals were found in mock-inoculated cells although using EM a yellow green autofluorescence was found in the cell wall of endocarp tissue (3I). Fluorescent spots were also found in lateral vascular bundles using EM (3J and 3K). CLSM analysis of the silique base exposed fluorescent cells in lateral vascular bundles (3L and 3M) and sometimes in or on parenchymatic tissues, possibly due to smearing during sample preparation (3M). In one case large group of fluorescent cells (a biofilm?) was found attached to exterior of the endocarp near to the replum (3N).

In the middle part of the silique, similar observations were done (4A-4E). In the replum fluorescent spots were found (4A, 4B, 4C and 4D) using EM which were also found using CLSM (4E). In addition, fluorescent spots were found in endocarp tissue near to the replum (4D).

In the top of the silique high bacterial densities were found in the replum using EM, but also at the transition of replum to septum. (4F and 4G). In mock-inoculated plants no typical GFP fluorescent spots were found (4H).

Discussion

The growth of a Brassica seed crop in the open field bears a high risk for the occurrence of seed infections with Xcc. Inoculum can be present on leaves of an infected Brassica plants nearby the seed production fields (Silva et al., 2017), on alternative host plants including weeds (Krauthausen et al., 2011), and in crop debris in or on soil (Köhl et al., 2011). Transmission of the pathogen from the inoculum sources may occur via aerosols, water splashes or by insects (Kuan et al., 1986, Poplawsky & Chun, 1998). In particular, pollinating insects may play a role in the occurrence of seed infections. They can pick up or release the bacterial pathogen while collecting the nectar produced by nectaries and thus inadvertently spread the pathogen, Deposition of cells on floral tissues will easily result in seed infections (van der Wolf & van der Zouwen, 2010, van der Wolf et al., 2013, Johnson et al., 1993).

Xcc may also be deposited on leaves or on the curd before flowering. It is still unknown if Xcc is able to cause seed infections after migration of the pathogen via vascular tissues in leaves, stems, peduncles, pedicels, siliques into the seed. These studies aimed to investigate the movement of the pathogen from leaf to seed by dividing its journey in trajectories. For these studies, vernalization-independent cauliflower plants were used to make multiple experiments in the project period of four years possible. Our hypothesis was that the risks for seed infections after leaf inoculation was small. The hypothesis was based on two observations. Firstly, companies observe that Xcc-free seed is frequently harvested from a highly (blackrot) symptomatic crop. Secondly, in previous studies, inoculation of plants in an early plant stage did not result in seed infections, while inoculation of blossoms resulted in a high levels of seed infections.

Inoculation of petioles resulted in the typical formation of black veins in inoculated leaves but no translocation into stems or peduncles was found. A rapid senescence of the inoculated leaves resulting in leaf fall which may have prevented translocation in the stems. This was found in repeated experiments both with cultivar Balaka and cultivar Campos. No indications were found that leaf fall was influenced by the inoculation Xcc, although a hypersensitive response as a result of Xcc infections cannot be excluded. A rapid leaf fall was also found for mock-inoculated and non-inoculated petioles.

Spray-inoculation of young plants of both cultivars in the six-leaf stage caused a severe symptom development resulting in necrotic leaves and incidentally in the development of symptomatic, dark-colored vascular tissue of the main stem. Translocation of Xcc in the developing plants was found up to the top of the peduncles for 8% of the cv. Balaka plants and 33% of the for cv. Campos plants. Xcc was not detected in all inflorescences of an infected plant indicating an irregular distribution of the pathogen in the peduncles. The pedicels of infected peduncles, however, were all negative. In other experiments during these studies it was shown that after inoculation of rachises, pedicels located near to the inoculation point became infected. Likely Xcc arrived too late in the peduncles and may be in too low densities for migration into the pedicels. The situation may be different in biannual Brassica seed production crops which are vernalization dependent. We hypothesize that in these plants systemic Xcc infections may occur more easily after leaf infections. In the first year of plant growth may progress into the main stems, while in the second year, peduncles, pedicels and siliques may become infected.

Three independently conducted experiments were done with inoculation of rachises; one at WUR with the GFP tagged Xcc strain IPO4050, one at Syngenta and one at ENZA with a wild type strain of Xcc. The experiment at WUR showed that on average 28% of the siliques became symptomatic. Xcc was found in a relatively high percentage of pedicels, siliques, septa, seed washings and seed extracts, after disinfection of the seeds with ethanol. This exhibited an efficient migration of Xcc during the

flowering phase from the rachises infected with high densities to seeds, in which a deep-seated infection was found at 60-80 dpi. The efficient transmission from rachises to seed was confirmed by the experiment conducted at Syngenta. Pedicels of Xcc-inoculated plants were positive in the dilution-plating assay but surprisingly most siliques were negative. A high percentage of the seed washings was positive (2.9%) often with high densities of Xcc, and also a high percentage (1%) of the extracts of warm-water treated seeds, indicating deep-seated infections. Similarly at ENZA, inoculation of the rachises resulted in an efficient translocation to pedicel, siliques and seeds. All pedicels, most siliques and 9.4% of the seeds, extracted without prior disinfection were positive in the dilution-plating assay.

In 2019, experiments with the GFP-tagged Xcc strain IPO4050 with a GFP expressing gene integrated in the amylase locus on the chromosome, indicated that the fluorescent signal was weak and difficult to observe using epifluorescence stereomicroscopy, even in highly infected plant tissue and even if the plant tissue was incubated on blotters with a semi-selective broth to enhance multiplication of the pathogen. The fluorescence intensity of the GFP-tagged strain IPO3555 harboring GFP on a replicating plasmid was higher and therefore we decided to use this strain in experiments with pedicel inoculation, despite its instability in GFP-expression. In experiments only requiring a short period between inoculation and analysis most Xcc cells still expresses GFP.

The fluorescence signal in tissues was higher on blotters drenched with TBY supplemented with starch than without. This enhancement of the fluorescence signal was found for IPO4050, for which GFP expression is under the expression of a amylase promotor, but also for IPO3555 which the GFP expression is not affected by starch. This indicated that the enhanced fluorescence was at least partially based on a higher multiplication rate of the GFP-tagged Xcc strains as a result of the supplement of starch and not only on an increased GFP expression due to an upregulation of the amylase promotor.

In 2020, experiments were done with inoculated pedicels, injected near to the proximal end of the siliques with IPO3555. It resulted in infection of the basis of all siliques attached to the inoculated pedicels whereas 90% of the top of the silique were infected, often with high densities. Infections resulted also in the development of black/brown lesions on siliques and/or in a necrotic tip. Unfortunately, siliques did not yield seed as cv. Campos only produced seed after manual pollination; the use of bumble bees was ineffective. Using fluorescence microscopy and confocal laser scanning bacteria a were found mainly in vascular tissue and the parenchyma cells of the replum of the siliques. Incidentally, Xcc was located in the septum or found in biofilms attached to the endocarp.

Microscopic studies in the past indicated that inoculation of pedicels with Xcc can result in colonization of the xylem tissue of the funicles, suggesting a translocation via the vascular system into the seed (Cook et al., 1952). In their studies, however, the transmission from funicles into the seed was never fully evidenced. Seed infections, however, may also be a result of contact with severely infected tissues of the valves or septum. In our studies, we found a specific GFP-signal both on the outer and inner tissues of the valves.

In conclusion, we found that after early infections of petioles of individual leaves, transmission of Xcc into stems is limited as a result of leaf fall due to ageing and possibly symptom development. Xcc, however could migrate from spray-inoculated young plants into the distal end of the peduncles. Although population densities in the peduncles could be high, no infections in the pedicels were found. Possibly, migration of Xcc into peduncles and/or the development of populations in peduncles started too late for migration into pedicels. If high densities of Xcc were inoculated in the rachises of peduncles, Xcc was able to migrate into pedicel nearby the inoculation point and further into siliques and seeds with a high efficiency, even resulting in deeper seated seed infections. Inoculation of rachises resulted in severe symptom development in a high percentage of the siliques. Studies with fluorescence microscopy indicated that after pedicel inoculations Xcc migrated via the vascular bundle of the suture. A strong signal in the suture of the silique is an indication for this systemic movement of the pathogen (van der Wolf et al., 2019). Seeds may have become infected via vascular tissue in septum and funicle, but also via contact with Xcc growing as a biofilm on the endocarp of the valves of the siliques.

Acknowledgement
This research was funded by the Dutch Ministry of Economic Affairs in the Topsector Program "Horticulture and Starting Materials" via the project "Routes van Xanthomonas campestris pv. campestris die resulteren in zaadinfecties in Brassica in kaart brengen" (grant nr. KV1509-044) and by the private partners Bejo Zaden B.V., Syngenta Seeds B.V., ENZA Zaden Seed Operations B.V. and Rijk Zwaan Breeding B.V. The authors are very grateful to Unifarm (Wageningen UR) for their excellent support in the cultivation of plant material.

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Supplement 1. Syngenta (2020)

Rachis inoculation Cauliflower Campos with Xcc strain IPO3078

Project: 2000	**Start: 2019-36**	**Author: Bernadette Kroon**
Exp. number: 2000-03	**Finish: 2020-20**	**Contributor(s):**

Key words
Xcc, IPO3078, cauliflower, rachis inoculation, external seed infection, internal seed infection

Objective(s)	**Result(s)**
Rachis inoculation of cauliflower cultivar Campos with Xcc strain IPO3078. Analyze pedicels, siliques and seeds for Xcc infection	• On average 2.3 seeds/silique were harvested from Xcc inoculated plants. • 14 out of 20 pedicels were infected with Xcc. Colony forming units (cfus) Xcc ranged from 96 till 5.1 x 10^5 /pedicel. Xcc was detected in just 2 of the corresponding siliques. • 32 of the 50 seed samples of Xcc inoculated plants were externally infected with Xcc ranging from 14 to 700000 cfu/35 seeds. 15 of these external positive samples were also internal infected.

Materials

Cauliflower seeds Campos (BEJO)	
Flowers of cauliflower 9395 (4374-2), 9396 (4374-1) and 2070 for pollination	
Xcc strain IPO3078	
Xcc strain ZUM2030	
TSA medium	
YDC medium	
CS20ABN medium	Appendix 4
PBS	
PBST	
1 ml syringe with needle (0.4 x 19)	
Grinder with ring head with ball bearings	• Hand grinder Bioreba (cat no. 400010) • Tecan type device (purpose built)
Water bath	52 °C
Bioreba Extraction bags Universal	12x15 cm, cat. no. 430100

Method

The protocol of Jan van der Wolf (WUR) (Appendix 1) was followed with some deviations.

Plants

Campos seeds were sown 3 September 2019. Two weeks after sowing, 20 Campos plants were transferred to 3 l pots (21 °C, 70% RH).

After 12 weeks one plant stayed in the greenhouse at 21 °C to observe if self-pollination could occur. The other plants were transferred to a climate cell at 5 °C to inhibit flowering.

As self-pollination did not occur, the other plants were pollinated manually (Table 1). Six till 20 days (Table 1) before pollination plants were transferred from the 5 °C climate cell to the greenhouse (25 °C, 75% RH). Due to fungi in the plants, affected parts of the plants were removed, plants were sprayed with Luna Sensation (BayerCrop Science) and RH was lowered to 50% on 29-1-2020. Unfortunately, thrips were introduced in the greenhouse by the flowers used for pollination. To

suppress thrips, Swirski-mite Plus (Koppert) was used and plants were sprayed with Vertimec (Syngenta). After the detection of thrips new rachises were removed as much as possible.

Inoculation
Xcc strain IPO3078 was grown on TSA medium. Three-days-old plates were used to make a suspension in PBS. The suspension was plated on NA medium to determine the concentration Xcc (approximately 10^8 cfu/ml, Table 1). Four days after pollination, rachises were inoculated with this suspension using a 1 ml syringe with needle (0.4 x 19). It was not possible to inject the suspension. A puncture was made with the needle and a small droplet was left at the puncture site (Figure 1). After a few minutes the drops were absorbed by the stem. Rachises were inoculated between two pedicels below the flowers that were pollinated. Control plants were inoculated with PBS. Plants 1, 2 and 3 were kept dry the day before inoculation (advice of Jan van der Wolf). However, as these plants were very bad at the moment of inoculation, the other plants were only kept dry at the day of inoculation. Water was given half an hour after the inoculation.

Table 1 *Scheme of pollination of flowers, inoculation of rachises and harvest of seeds. 11 plants were inoculated with Xcc IPO3078, 3 plants were inoculated with PBS (control).*

Plant	Transferred to 25 °C	pollination	# flowers pollinated	Male line	Concentration Xcc in suspension used for inoculation	Harvest of seeds (days after inoculation)
1	10-1-2020	16-1-2020	176	9395 (4374-2)	Xcc (4.2 x 10^8 cfu/ml)	35-43
2	10-1-2020	16-1-2020	49	9395 (4374-2)	Xcc (4.2 x 10^8 cfu/ml)	35-46
3	10-1-2020	16-1-2020	35	9395 (4374-2)	Control (PBS)	35-46
4	10-1-2020	23-1-2020	183	9396 (4374-1)	Xcc (3.1 x 10^8 cfu/ml)	28-43
5	10-1-2020	23-1-2020	79	2070	Control (PBS)	31-42
6	10-1-2020	23-1-2020	137	9396 (4374-1)	Xcc (3.1 x 10^8 cfu/ml)	28-43
7	14-1-2020	23-1-2020	106	2070	Xcc (3.1 x 10^8 cfu/ml)	28-43
8	10-1-2020	30-1-2020	104	9396 (4374-1)	Xcc (3.5 x 10^8 cfu/ml)	28-42
9	10-1-2020	30-1-2020	76	2070	Control PBS	35-42
10	10-1-2020	30-1-2020	62	9396 (4374-1)	Xcc (3.5 x 10^8 cfu/ml)	28-42
11	17-1-2020	30-1-2020	20	2070	Xcc (3.5 x 10^8 cfu/ml)	28-42
12	17-1-2020	30-1-2020	16	2070	Xcc (3.5 x 10^8 cfu/ml)	28-42
13	17-1-2020	30-1-2020	39	2070	Xcc (3.5 x 10^8 cfu/ml)	28-42
14	17-1-2020	30-1-2020	174	9396 (4374-1)	Xcc (3.5 x 10^8 cfu/ml)	28-42

Pathogenicity of strain IPO3078
Pathogenicity of strain IPO3078 was tested on cotyledons of 7 days-old- plants (white cabbage E45D0508, batch 11439075). As controls Xcc strain ZUM2030 and NB medium were used. The Xcc isolates were incubated for 24 h in 1 ml NB in Eppendorf tubes on a shaker at room temperature. Twenty plants per treatment were inoculated by puncturing the main vein of both cotyledons with a toothpick dipped in the suspension, while the cotyledon was supported by a cotton swab that was also dipped in the suspension.

Analyses of pedicels, siliques and seeds
8 siliques with attached pedicels were collected 42 days after inoculation and 12 siliques were collected 45 days after inoculation (Table 3). To prevent cross-contamination between siliques hands and equipment (scalpel, forceps) were disinfected before handling the next silique. Pedicels and siliques were separated after removal of the seeds and put individually in a Bioreba bag. 800 µl PBST was added to the bags with the pedicels and 1000 µl to the bags with the siliques. Pedicels were ground manually with a Bioreba tissue homogenizer consisting of a circular array of steel balls. The siliques were ground with a Tecan grinder. Undiluted and 100 times diluted extract was plated on CS20ABN medium. The plates were incubated for 72 h at 26°C.

28-46 days (Table 1) after inoculation seeds were harvested from ripe siliques and stored in paper bags at 12 °C and 32% RH. Seeds were pooled per plant and cross-contamination between seeds of different plants was prevented.

50 subsamples of 35 seeds of Xcc inoculated siliques and 10 subsamples of 30 seeds of PBS inoculated siliques were put in 2.2 ml Eppendorf tubes (Table 2).

Figure 1 *Droplets of Xcc suspension at puncture site.*

0.7 ml of PBST was added to 35 seeds samples and 0.6 ml was added to 30 seeds samples. Samples were shaken for 10 min at room temperature. Undiluted and 100 x diluted washing solution was plated on CS20ABN medium. Xcc strains IPO3078 and ZUM2030 were plated as references. The plates were incubated for 72 h at 26°C. Seeds were O/N air dried on filter papers in Petri dishes and stored at 11°C and 50% RH until the results of the assessment of the external contaminations were known.

Only samples positive for external contamination were processed for internal infections. External Xcc contamination was removed using a 10 min 52 °C treatment. Positive samples were put in Bioreba bags and 1 ml PBST at 52 °C was added to the bags. The bags were placed for 10 minutes in a 1000 ml volume beaker containing water of 52 °C which was placed in a water bath previously set at 52 °C. After 10 minutes the bags were cooled in a beaker with cold water. The seeds were ground in the PBST with a Tecan grinder. Undiluted, 100- and 1000-times diluted extract was plated on CS20ABN medium. The plates were incubated for 7 days at 26 °C.

Table 2 *Number of subsamples of 35 seeds (Xcc) and 30 seeds (control).*

Plant	Inoculation	Xcc/control	Subsamples
1	20-1 2020	Xcc	7
2	20-1 2020	Xcc	2
4	27-1-2020	Xcc	8
6	27-1-2020	Xcc	8
7	27-1-2020	Xcc	8
8	3-2-2020	Xcc	5
10	3-2-2020	Xcc	2
11	3-2-2020	Xcc	1
12	3-2-2020	Xcc	0
13	3-2-2020	Xcc	0
14	3-2-2020	Xcc	9
3	20-1 2020	control	1
5	27-1-2020	control	4
9	3-2-2020	control	5

Results

Symptoms

From 4 days after inoculation brown discoloration was visible at the points of Xcc inoculation, sometimes resulting in brown stripes on the rachis (Figure 2). Rachises inoculated with PBS did not show any reaction. Irregular shaped black necrotic spots sometimes developed on siliques above Xcc inoculation spots (Figure 3).

Rachises, Xcc as well as PBS inoculated ones, sometimes developed black tops.

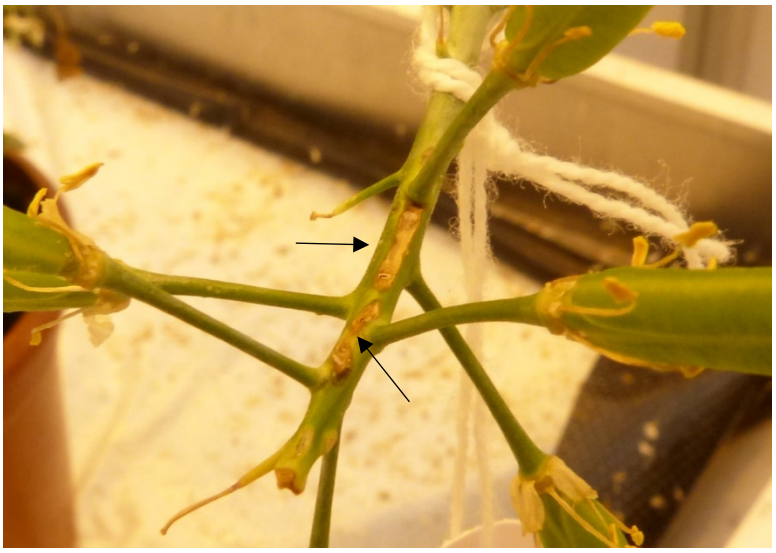

Figure 2 *Brown stripes on rachis at Xcc inoculation site.*

Figure 3 *Necrotic spots on silique.*

Infection of pedicels and siliques

Raw data on pedicel and silique infection can be found in Appendix 2. Fourteen out of 20 pedicels, harvested 42 or 45 days after inoculation, were infected with Xcc. Colony forming units (cfus) Xcc ranged from 96 till 5.1 x 10^5 /pedicel. Xcc was detected in just 2 of the corresponding siliques (Table 3).

Table 3 *Cfus Xcc and saprophytes detected in 20 pedicels and siliques 42 or 45 days after inoculation of the rachis.*

Plant	Days after inoculation	cfu Xcc/pedicel	cfu Xcc/silique	cfu saprophytes/pedicel	cfu saprophytes/silique
6-1	42	512000	8800	0	0
6-2	42	100000	0	0	0
6-3	42	388800	0	0	0
6-4	42	340800	0	0	0
6-5	42	212000	0	0	0
7-1	45	0	0	0	910
7-2	45	0	0	0	0
7-3	42	2704	0	0	0
7-4	42	96	0	0	0
7-5	42	0	0	0	57000
4-1	45	0	0	0	0
4-2	45	128	0	0	0
4-3	45	427200	0	0	0
4-4	45	35200	0	0	0
4-5	45	79200	0	0	510
4-6	45	0	0	0	0
4-7	45	124800	0	0	14000
4-8	45	302400	0	0	38000
4-9	45	704000	250	0	0
4-10	45	0	0	0	1060

Infection of seeds

Number of Xcc inoculated siliques and seeds, harvested in week 4, 5 and 6 after inoculation, is summarized in Table 4. Data on control inoculated seeds can be found in Table 5. On average only 2.3 (Xcc) and 2.1 (control) seeds were isolated per silique.

Table 4 *Number of Xcc inoculated siliques and seeds per plant harvested in week 4, 5 and 6 after inoculation.*

plant	weeks after inoculation						SUM	
	4		5		6			
	siliques	seeds	siliques	seeds	siliques	seeds	siliques	seeds
1			85	274	8	1	93	275
2			10	52	32	39	42	91
4	17	28	62	145	76	125	155	298
6	40	76	86	231	21	11	147	318
7	6	21	74	272	19	26	99	319
8	10	34	29	86	57	75	96	195
10	7	6	44	92	5	4	56	102
11	7	9	7	28	4	10	18	47
12			6	15	10	15	16	30
13	4	3			15	15	19	18
14	6	9	118	260	34	62	158	331
SUM	97	186	521	1455	281	383	899	2024

Table 5 *Number of PBS (control) inoculated siliques and seeds per plant harvested in week 4, 5 and 6 after inoculation.*

plant	weeks after inoculation						SUM	
	4		5		6			
	siliques	seeds	siliques	seeds	siliques	seeds	siliques	seeds
3			5	26	15	13	20	39
5	3	1	59	106	9	17	71	124
9			37	100	25	60	62	160
SUM	3	1	101	232	49	90	153	323

Raw data on seed infection can be found in Appendix 3. Thirty-two of the 50 samples of Xcc inoculated plants were externally infected with Xcc ranging from 14 to 7 x 10^5 cfu/35 seeds. From 15 of these 32 samples, Xcc was also recovered after warm water treatment and grinding (Table 6). Only in samples with an external infection of at least 21700 cfu Xcc/35 seeds, internal infection could be determined (Table 6 and Figure 4). Xcc was not detected (external) in the samples of the PBS inoculated control plants.

Pathogenicity of strain IPO3078

Pathogenicity of Xcc strain IPO3078 was compared with pathogenicity of strain ZUM2030 by inoculating cotyledons of 20 7-day-old plants. Two weeks after inoculation all cotyledons of plants inoculated with Xcc showed symptoms. At that time 12 plants inoculated with ZUM 2030 showed systemic infection as shown by symptoms on the true leaves, whereas 4 plants inoculated with IPO3078 showed systemic infection (Table 6). Four weeks after inoculation all ZUM2030 inoculated plants and 13 IPO3078 plants showed systemic symptoms (Table 6 and Figure 5). All NB inoculated control plants were symptomless.

Table 6 *Cfus Xcc/35 seeds after soaking the seeds (external) and after warm water treatment followed by grinding (internal).*

sample	cfu Xcc/35 seeds external	cfu Xcc/35 seeds internal	sample	cfu Xcc/35 seeds external	cfu Xcc/35 seeds internal
1-1	466200	280	**7-1**	252700	1960
1-2	294	0	**7-2**	700000	40
1-3	42	0	**7-3**	700000	22000
1-4	0	nd	**7-4**	161	0
1-5	0	nd	**7-5**	21700	1010
1-6	0	nd	**7-6**	299600	210
1-7	0	nd	**7-7**	0	nd
2-1	168	0	**7-8**	0	nd
2-2	480200	940	**8-1**	21700	0
4-1	0	nd	**8-2**	12600	0
4-2	616000	70	**8-3**	488600	910000
4-3	35	0	**8-4**	0	nd
4-4	0	nd	**8-5**	0	nd
4-5	10500	0	**10-1**	0	nd
4-6	70700	0	**10-2**	63	0
4-7	161	0	**11-1**	0	nd
4-8	49	0	**14-1**	700000	344000
6-1	351400	2010	**14-2**	0	nd
6-2	0	nd	**14-3**	0	nd
6-3	508200	360	**14-4**	14	0
6-4	700000	210	**14-5**	0	nd
6-5	0	nd	**14-6**	245	0
6-6	319200	0	**14-7**	308	0
6-7	700000	62000	**14-8**	0	nd
6-8	369600	250	**14-9**	105	0

nd: not determined.

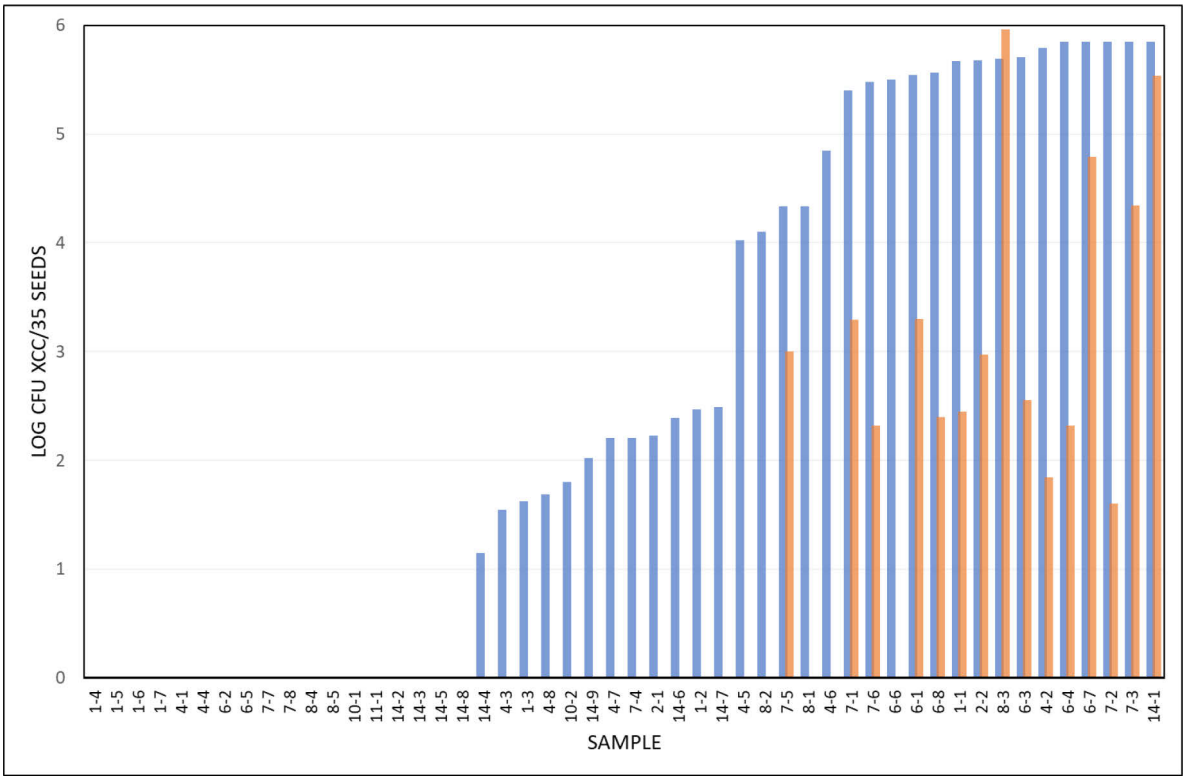

Figure 4 *Log cfu Xcc/35 seeds. Blue: external infection. Orange: internal infection. Note that samples showing no infection at all were only tested for external infection.*

The percentage of externally infected seeds was estimated at 2.9%, whereas the percentage of internal infections was estimated at 1%.

Table 6 *Number of plants with Xcc symptoms on true leaves, 14, 21 and 24 days after inoculation of the cotyledons of 20 plants with Xcc strain ZUM2030 or IPO3078 or with NB (control).*

Days after inoculation	ZUM 2030	IPO 3078	NB (control)
14	12	4	0
21	19	11	0
24	20	13	0

Figure 5 *Symptoms on IPO3078 inoculated plants (top) and ZUM2030 inoculated plants (bottom), 26 days after inoculation.*

Appendix.

Recipe mCS20ABN (per liter).

Part	Ingredient	Amount	Comment
A	Soya Peptone (Oxoid L44)	2.0 g	
A	$(NH_4)_2HPO_4$	0.8 g	
A	KH_2PO_4	2.8 g	
A	$MgSO_4 \cdot 7H_2O$	0.4 g	
A	Tryptone (Difco Bacto)	2.0 g	
A	L-Glutamine (Sigma G-3126)	6.0 g	
A	L-Histidine (Sigma H-8000)	1.0 g	
A	D-Glucose (Dextrose)	1.0 g	
A	Agar	18 g	
A	Soluble starch (Aldrich 17,993-0)	25.0 g	
A	Distilled/deionized water	1000 ml	
B	Cycloheximide	1 ml	200 mg/ml 70% ethanol
B	Neomycin	1 ml	40 mg/ml 20% ethanol
B	Bacitracin	2 ml	50 mg/ml 50% ethanol

Prepare part A.

Measure pH and adjust to pH 6.7.

Autoclave.

Allow medium to cool to approx. 50°C and add antibiotics (part B).

After autoclaving pH is usually 0.1 - 0.3 units lower. Measure pH of plates with a flat surface electrode. pH must be below 6.6.

Supplement 2. ENZA

PROJECT TITLE : Risk of seed infections due to (systemic) colonization of Xcc in Brassica

Contact SPR : Pauline Bernardo

Goals

- Determine the risk of seed infections due to (systemic) colonization of Brassica plants after inoculation of rachises with Xcc.

Deliverables

- Knowledge on Xcc risk of infection in Brassica seeds after inoculation of rachises.
- Sharing results/report with collaborators (PPS KV1509-044 / Jan van der Wolf).

Time planning

Table 1 *Planning of activities.*

Date	Activity/Observation
20-11-2019	Sowing.
4-12-2019	Transplanting.
15-2-2020	Curds are starting to appear.
09-13/03/2020	1st pollination week 11[a].
16-20/03/2020	2nd pollination week 12[a].
23-3-2020	Inoculation Xcc in rachises of pollinated flowers only[b].
31-3-2020	Changing conditions in greenhouse to raise relative humidity, wet floor, close vents.
1-4-2020	Reinoculation[b].
24-4-2020	Stop floor watering for pods to dry.
06-07/05/2020	Pods collection/sampling/plating.
11-5-2020	Plates analysis.
15-6-2020	Reporting.

[a] Plants were not at the same phenological stage; therefore, pollination was performed for two consecutive weeks.

[b] First inoculation seemed to have failed to induce infection because no blackening of inoculation point or stem one week after inoculation.

Introduction

*Xanthomonas campestris pv. campestris (*Xcc) is an important seed-borne bacterial pathogen in Brassica crops (Williams 1980). It can lead to black rot of crucifers which causes important crop losses.

Enza Zaden is currently involved in a PPS project lead by Jan van der Wolf (Wageningen University) aiming to: a/Gain insight into the role of contaminated pollen in the epidemiology of Xcc, b/Determine the risk of seed infections due to (systemic) colonization of Brassica plants after inoculation of leaf or rachis with Xcc. The knowledge will be used together with literature data and knowledge from practice for c/Defining so-called "critical control points" that help to reduce the risk of seed contamination.

In the present study, we assessed the risk of seed infection after inoculation of cauliflower rachises (part of aim "b").

Material and methods

- **Bacterial strain, buffer and culture media**

In the present experiment, Xcc wild type strain IPO 3078 was used. The strain stock was conserved in 30% glycerol at -80°C. Xcc inoculum was prepared from cultures grown on tryptone soya agar (TSA) at 25 °C for 48 h. Suspensions of Xcc were prepared in water.

- **Brassica plants and growth conditions**

The cauliflower hybrid Campos (Brassica oleracea) provided by Bejo Zaden was used in this experiment. Plants were grown from late November 2019 until late April 2020 (Table 1) in a greenhouse with a recorded average temperature of 20°C (minimum 16°C, maximum 29°C). Hand pollination was performed from the 9th to the 20th of March. Relative humidity (RH) was increased by watering the flour and closing vents starting April 1st (RH variating from 60 to 100%). Siliques were collected per treatment in plastic ziploc back when maturity was reached (6th of May) and Xcc testing was performed immediately or the day after collection. Between different plants sampled, the equipment used was disinfected with 70% ethanol and gloves were changed.

- **Xcc inoculation**

Inoculations of Xcc suspension (OD~0.1) was performed with a syringe and needle the 23rd of April in every rachis of flower stalk that were hand pollinated (Figure 1). Since no disease development was observed after a week on the inoculation point, humidity was raised as described above and inoculation was performed again a on the 1st of April. Ten plants were inoculated with Xcc and three with water (negative control).

Figure 1 *Rachis inoculation with syringe and needle.*

- **Black rot incidence of siliques**

The occurrence of black rot in peduncles, siliques and seeds was assessed at 5 months after potting which was 35 days after the last/final inoculation. Twenty siliques were collected for each treatment, therefore 2 siliques per infected plant were collected, and 7 or 6 per water inoculated plants. For the plants inoculated with Xcc, the focus was on collecting symptomatic siliques with a seed set. Pedicel, siliques and seeds were processed separately. While pedicel and siliques were tested individually, seeds were pooled per treatment and divided in 50 subsamples. Organs collected were ground in PBST (PBS 0.05M + 0.02% Tween20, 0.5mL for the pedicel, 1mL for seeds and silliques). An aliquot of 100 µL of undiluted,100 and 10000 times diluted extracts were plated on mFS. Plates were incubated at 25°C for 5 days and Xcc suspected colonies (starch hydrolysing) counted.

- **Infected seed incidence estimation**

The estimation of infected seed incidence was calculated using the online tool https://epitools.ausvet.com.au/ppvariablepoolsize. Briefly, the method is using generalized linear modelling to calculate maximum-likelihood estimates of prevalence and confidence limits where multiple different pool sizes are used (Williams & Moffitt, 2001). The method assumes 100% test sensitivity and specificity.

Results

Inoculation of rachises resulted in approximately 50% of symptomatic siliques close to the points of inoculation (Figure 2, top). In this experiment, for Xcc inoculated plants, only symptomatic siliques were sampled for assessment of the development of the disease in pedicel, siliques and seeds (Figure 2, bottom right).

Samples harvested from water inoculated plants remained free from Xcc (Table 2). All the collected pedicels and all except one silique from inoculated plants tested positive for Xcc (Table 2, Annex 1). A total of 133 seeds were collected from negative control siliques (average of 6.7 seeds/silique), and 180 for symptomatic siliques (average of 9 seeds/silique). Fifteen pools of seeds over a total of fifty resulted positive, leading to an estimated infected seed prevalence of 9.47% with CI95%=[5.55-14.71].

Table 2 *Proportion of Xcc infected samples.*

Treatment	Pedicels	Silliques	Pools of seeds
Water	0/20	0/20	0/50
Xcc	20/20	19/20	15/50

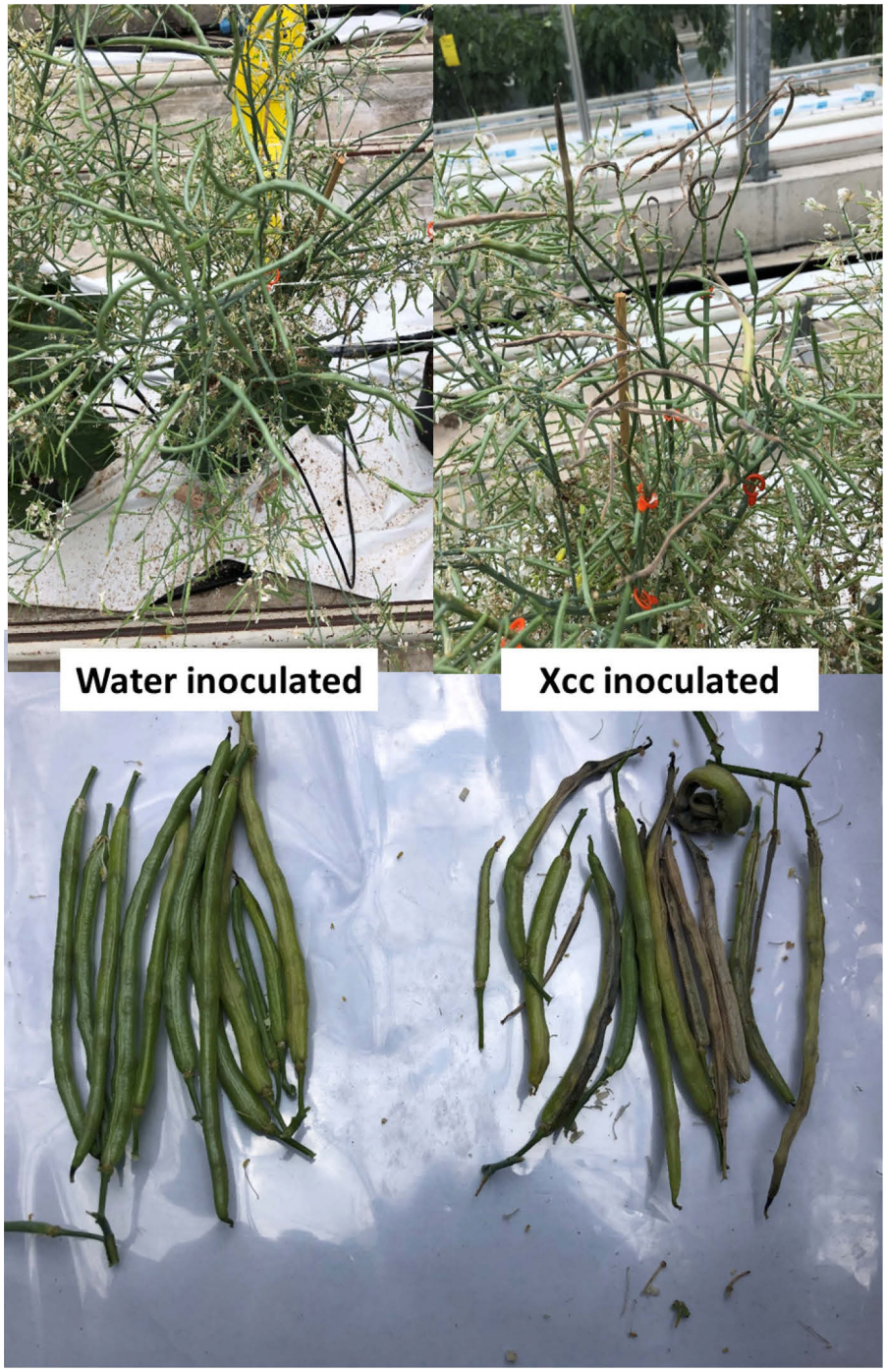

Figure 2 *Siliques of water vs. Xcc inoculated plants.*

Conclusion and discussion

In the present experiment, our goal was to investigate the propagation of Xcc to seeds after rachis inoculation. With the method described in this report, all of the pedicels and all but one pod got infected and we obtained a prevalence of Xcc infected seeds of 9.47% with CI95%=[5.55-14.71], which is substantially high. This demonstrates that the pathogen is able to translocate from the rachis to the seeds. It is important to note that those results have been obtained from one hybrid. It will therefore still be valuable to confirm those results in different parental lines used for seed production.

As a side note, we saw that the number of seeds obtained from water control plants was lower than the infected ones (133 vs. 180) for a same number of examined siliques. This is most likely a result of different people harvesting different treatments (an intern collected the negative control siliques while the researcher collected the infected ones. It seems that therefore there is a "sampler" biais here that could have influenced the number of seeds obtained.)

References

Williams, P. H. (1980). Black rot: A continuing threat to world crucifers. Plant Disease, 64, 736–742.

Williams CJ, Moffitt CM, 2001. A critique of methods of sampling and reporting pathogens in populations of fish. Journal of Aquatic Animal Health 13: 300-309.

Annex 1 ***Details of Xcc CFUs count on MFs.*** *NC=not countable, positive samples highlighted in orange.*

Sample#	Sillique #	Organ	#seeds	CFUs Undiluted	CFUs dil x100	CFUs dil x10000
1	Water 1	Pedicel	NA	0	0	0
2	Water 2	Pedicel	NA	0	0	0
3	Water 3	Pedicel	NA	0	0	0
4	Water 4	Pedicel	NA	0	0	0
5	Water 5	Pedicel	NA	0	0	0
6	Water 6	Pedicel	NA	0	0	0
7	Water 7	Pedicel	NA	0	0	0
8	Water 8	Pedicel	NA	0	0	0
9	Water 9	Pedicel	NA	0	0	0
10	Water 10	Pedicel	NA	0	0	0
11	Water 11	Pedicel	NA	0	0	0
12	Water 12	Pedicel	NA	0	0	0
13	Water 13	Pedicel	NA	0	0	0
14	Water 14	Pedicel	NA	0	0	0
15	Water 15	Pedicel	NA	0	0	0
16	Water 16	Pedicel	NA	0	0	0
17	Water 17	Pedicel	NA	0	0	0
18	Water 18	Pedicel	NA	0	0	0
19	Water 19	Pedicel	NA	0	0	0
20	Water 20	Pedicel	NA	0	0	0
21	XCC 1	Pedicel	NA	NC	1	0
22	XCC 2	Pedicel	NA	NC	60	1
23	XCC 3	Pedicel	NA	NC	27	0
24	XCC 4	Pedicel	NA	NC	18	0
25	XCC 5	Pedicel	NA	NC	8	0
26	XCC 6	Pedicel	NA	NC	5	0
27	XCC 7	Pedicel	NA	NC	39	0
28	XCC 8	Pedicel	NA	NC	3	0
29	XCC 9	Pedicel	NA	NC	2	0
30	XCC 10	Pedicel	NA	66	0	0
31	XCC 11	Pedicel	NA	NC	19	2
32	XCC 12	Pedicel	NA	NC	7	0
33	XCC 13	Pedicel	NA	NC	14	44
34	XCC 14	Pedicel	NA	NC	35	0
35	XCC 15	Pedicel	NA	NC	32	2

Sample#	Sillique #	Organ	#seeds	CFUs Undiluted	CFUs dil x100	CFUs dil x10000
36	XCC 16	Pedicel	NA	NC	1	0
37	XCC 17	Pedicel	NA	44	1	0
38	XCC 18	Pedicel	NA	NC	6	3
39	XCC 19	Pedicel	NA	NC	13	0
40	XCC 20	Pedicel	NA	80	2	0
41	Water 1	Sillique	NA	0	0	0
42	Water 2	Sillique	NA	0	0	0
43	Water 3	Sillique	NA	0	0	0
44	Water 4	Sillique	NA	0	0	0
45	Water 5	Sillique	NA	0	0	0
46	Water 6	Sillique	NA	0	0	0
47	Water 7	Sillique	NA	0	0	0
48	Water 8	Sillique	NA	0	0	0
49	Water 9	Sillique	NA	0	0	0
50	Water 10	Sillique	NA	0	0	0
51	Water 11	Sillique	NA	0	0	0
52	Water 12	Sillique	NA	0	0	0
53	Water 13	Sillique	NA	0	0	0
54	Water 14	Sillique	NA	0	0	0
55	Water 15	Sillique	NA	0	0	0
56	Water 16	Sillique	NA	0	0	0
57	Water 17	Sillique	NA	0	0	0
58	Water 18	Sillique	NA	0	0	0
59	Water 19	Sillique	NA	0	0	0
60	Water 20	Sillique	NA	0	0	0
61	XCC 1	Sillique	NA	0	0	0
62	XCC 2	Sillique	NA	NC	1	0
63	XCC 3	Sillique	NA	NC	34	0
64	XCC 4	Sillique	NA	5	0	0
65	XCC 5	Sillique	NA	NC	7	0
66	XCC 6	Sillique	NA	NC	NC	11
67	XCC 7	Sillique	NA	NC	NC	7
68	XCC 8	Sillique	NA	NC	36	1
69	XCC 9	Sillique	NA	NC	NC	8
70	XCC 10	Sillique	NA	NC	20	1
71	XCC 11	Sillique	NA	NC	NC	13
72	XCC 12	Sillique	NA	NC	NC	13
73	XCC 13	Sillique	NA	NC	58	4
74	XCC 14	Sillique	NA	NC	76	0
75	XCC 15	Sillique	NA	NC	NC	5
76	XCC 16	Sillique	NA	NC	8	3
77	XCC 17	Sillique	NA	NC	3	0
78	XCC 18	Sillique	NA	NC	NC	1
79	XCC 19	Sillique	NA	NC	63	0
80	XCC 20	Sillique	NA	NC	NC	17
81	WATER	Pool seeds 1	3	0	0	0
82	WATER	Pool seeds 2	3	0	0	0
83	WATER	Pool seeds 3	3	0	0	0
84	WATER	Pool seeds 4	3	0	0	0
85	WATER	Pool seeds 5	3	0	0	0
86	WATER	Pool seeds 6	3	0	0	0
87	WATER	Pool seeds 7	3	0	0	0
88	WATER	Pool seeds 8	3	0	0	0
89	WATER	Pool seeds 9	3	0	0	0
90	WATER	Pool seeds 10	3	0	0	0
91	WATER	Pool seeds 11	3	0	0	0
92	WATER	Pool seeds 12	3	0	0	0
93	WATER	Pool seeds 13	3	0	0	0

Sample#	Sillique #	Organ	#seeds	CFUs Undiluted	CFUs dil x100	CFUs dil x10000
94	WATER	Pool seeds 14	3	0	0	0
95	WATER	Pool seeds 15	3	0	0	0
96	WATER	Pool seeds 16	3	0	0	0
97	WATER	Pool seeds 17	3	0	0	0
98	WATER	Pool seeds 18	3	0	0	0
99	WATER	Pool seeds 19	3	0	0	0
100	WATER	Pool seeds 20	3	0	0	0
101	WATER	Pool seeds 21	3	0	0	0
102	WATER	Pool seeds 22	3	0	0	0
103	WATER	Pool seeds 23	3	0	0	0
104	WATER	Pool seeds 24	3	0	0	0
105	WATER	Pool seeds 25	3	0	0	0
106	WATER	Pool seeds 26	3	0	0	0
107	WATER	Pool seeds 27	3	0	0	0
108	WATER	Pool seeds 28	3	0	0	0
109	WATER	Pool seeds 29	3	0	0	0
110	WATER	Pool seeds 30	3	0	0	0
111	WATER	Pool seeds 31	3	0	0	0
112	WATER	Pool seeds 32	3	0	0	0
113	WATER	Pool seeds 33	3	0	0	0
114	WATER	Pool seeds 34	2	0	0	0
115	WATER	Pool seeds 35	2	0	0	0
116	WATER	Pool seeds 36	2	0	0	0
117	WATER	Pool seeds 37	2	0	0	0
118	WATER	Pool seeds 38	2	0	0	0
119	WATER	Pool seeds 39	2	0	0	0
120	WATER	Pool seeds 40	2	0	0	0
121	WATER	Pool seeds 41	2	0	0	0
122	WATER	Pool seeds 42	2	0	0	0
123	WATER	Pool seeds 43	2	0	0	0
124	WATER	Pool seeds 44	2	0	0	0
125	WATER	Pool seeds 45	2	0	0	0
126	WATER	Pool seeds 46	2	0	0	0
127	WATER	Pool seeds 47	2	0	0	0
128	WATER	Pool seeds 48	2	0	0	0
129	WATER	Pool seeds 49	2	0	0	0
130	WATER	Pool seeds 50	2	0	0	0
131	XCC	Pool seeds 1	4	1	0	0
132	XCC	Pool seeds 2	4	1	0	0
133	XCC	Pool seeds 3	4	0	0	0
134	XCC	Pool seeds 4	4	1	0	0
135	XCC	Pool seeds 5	4	1	0	0
136	XCC	Pool seeds 6	4	0	0	0
137	XCC	Pool seeds 7	4	0	0	0
138	XCC	Pool seeds 8	4	0	0	0
139	XCC	Pool seeds 9	4	1	0	0
140	XCC	Pool seeds 10	4	0	0	0
141	XCC	Pool seeds 11	4	0	0	0
142	XCC	Pool seeds 12	4	24	0	0
143	XCC	Pool seeds 13	4	47	0	0
144	XCC	Pool seeds 14	4	0	0	0
145	XCC	Pool seeds 15	4	0	0	0
146	XCC	Pool seeds 16	4	0	0	0
147	XCC	Pool seeds 17	4	0	0	0
148	XCC	Pool seeds 18	4	0	0	0
149	XCC	Pool seeds 19	4	0	0	0
150	XCC	Pool seeds 20	4	1	0	0
151	XCC	Pool seeds 21	4	5	0	0

Sample#	Sillique #	Organ	#seeds	CFUs Undiluted	CFUs dil x100	CFUs dil x10000
152	XCC	Pool seeds 22	4	0	0	0
153	XCC	Pool seeds 23	4	1	0	0
154	XCC	Pool seeds 24	4	0	0	0
155	XCC	Pool seeds 25	4	0	0	0
156	XCC	Pool seeds 26	4	0	0	0
157	XCC	Pool seeds 27	4	0	0	0
158	XCC	Pool seeds 28	4	0	0	0
159	XCC	Pool seeds 29	4	0	0	0
160	XCC	Pool seeds 30	4	0	0	0
161	XCC	Pool seeds 31	3	0	0	0
162	XCC	Pool seeds 32	3	0	0	0
163	XCC	Pool seeds 33	3	NC	0	0
164	XCC	Pool seeds 34	3	0	0	0
165	XCC	Pool seeds 35	3	0	0	0
166	XCC	Pool seeds 36	3	1	0	0
167	XCC	Pool seeds 37	3	0	0	0
168	XCC	Pool seeds 38	3	4	0	0
169	XCC	Pool seeds 39	3	2	0	0
170	XCC	Pool seeds 40	3	0	0	0
171	XCC	Pool seeds 41	3	0	0	0
172	XCC	Pool seeds 42	3	63	0	0
173	XCC	Pool seeds 43	3	0	0	0
174	XCC	Pool seeds 44	3	0	0	0
175	XCC	Pool seeds 45	3	0	0	0
176	XCC	Pool seeds 46	3	0	0	0
177	XCC	Pool seeds 47	3	0	0	0
178	XCC	Pool seeds 48	3	0	0	0
179	XCC	Pool seeds 49	3	0	0	0
180	XCC	Pool seeds 50	3	0	0	0
PC	IPO3078	Normal growth blue green translucent colonies with halo				
Blank	PBST 0,05M	No growth				

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Report WPR-1028

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The mission of Wageningen University & Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 6,500 employees (5,500 fte) and 12,500 students, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.