

Impact of bacterial spot outbreaks on the phytosanitary quality of tomato and pepper seeds

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The impact of disease outbreaks on the phytosanitary quality of seeds was investigated for two pathosystems: tomato–*Xanthomonas vesicatoria* and pepper–*Xanthomonas euvesicatoria*. This study, which was performed in Italy and Serbia, aimed to evaluate the season-to-season transmission of phytopathogenic regulated bacteria associated with phytosanitary risks posed by seeds produced in areas where bacterial infections are possible. For each pathosystem, field plots were experimentally inoculated to simulate an initial infection rate of 1%, 5% and 15%. The area under the disease progress curve (AUDPC) was calculated for each field plot, the seeds produced were analysed to determine the contamination level and rate, and the plant-to-seed transmission was evaluated by a seedling grow-out (SGO) assay. To investigate transmission under field conditions, a second-year experiment was performed, wherein seeds collected from the first year were used to establish new field plots. During the first growing season, AUDPC values were positively correlated with the percentages of initial infection for each pathosystem. Seed contamination levels in pepper ranged from 34 to 100 CFU g⁻¹, and the contamination rate ranged from 1.50% up to 3.17% for *X. euvesicatoria*, whereas processing and fresh market tomato seeds produced both in Italy and Serbia were not infected by *X. vesicatoria*. During SGO assays and the second cropping year, no symptoms were observed in either tomato or pepper plants. Therefore, the calculated pepper seed contamination rate for *X. euvesicatoria* appeared to be less than the threshold necessary to initiate a disease outbreak. Finally, all seeds obtained during the second cropping year were uninfected.

Keywords: bacterial leaf spot, pathogen transmission, phytosanitary seed quality, seedborne bacteria

Introduction

Infected seeds are the most important pathway for the introduction and spread of several plant pathogenic bacteria that may affect both fruit quality and plant viability, thus causing economic losses worldwide (Gitaitis & Walcott, 2007). Among other bacterial diseases, tomato can be affected by bacterial spot caused by *Xanthomonas vesicatoria* (Xv; Doidge, 1921; Vauterin *et al.*, 1995) and *X. euvesicatoria* (Xe; Jones *et al.*, 2004). The latter is particularly aggressive against pepper (Ignjatov *et al.*, 2010). These bacteria are spread through contaminated/infected seeds as the primary source of inoculum (Dutta *et al.*, 2014) and are listed as quarantine organisms by the European Plant Protection Organization (EPPO). These pathogens can survive in seeds for extended periods (Bashan *et al.*, 1982a); such survival ability allows long-distance dissemination and facilitates their introduction into pathogen-free areas through seed trade. Seed testing and certification (EPPO, 2013) and seed production in pathogen-free areas with no conducive

environmental conditions, are the most important management strategies for the preventive control of these pathogens. Sanitation of potentially contaminated seeds is only partially achieved using common disinfection methods, e.g. heat treatment, fermentation of fruit pulp in the case of tomato (Chambers & Merriman, 1975; Dhanvantari, 1989), or chlorine/acid treatment for pepper seeds (Dempsey & Walker, 1973). So far, there is no method available that can ensure the complete eradication of pathogens from naturally infected seeds without dramatically reducing seed germination (Dhanvantari, 1989). Despite the use of phytosanitary certification and quarantine procedures in domestic and international seed trade, which can considerably reduce disease incidence, severe epidemics are occasionally reported (Gitaitis & Walcott, 2007).

Transplant production studies on the transmissibility of *Clavibacter michiganensis* subsp. *michiganensis* (Strider, 1969; Davis *et al.*, 1984), the causal agent of tomato bacterial canker, revealed that a single infected tomato seed among 10 000 is sufficient to initiate an epidemic under favourable conditions (Chang *et al.*, 1991). Similar studies are currently not available for Xv or Xe. Very few studies have been conducted under field conditions to evaluate the seed contamination threshold necessary for pathogen transmission from seed to plants. Chang *et al.* (1991) demonstrated a systemic infection in

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tomato transplants grown from infected seeds containing approx. 10^4 CFU g^{-1} of *C. michiganensis* subsp. *michiganensis*. However, knowledge regarding the Xv and Xe seed contamination thresholds needed for disease expression on tomato and pepper plants under field conditions is lacking. Bacterial spot is a polycyclic disease. Secondary inocula might be abundantly produced during the cropping period and may play a key role in the short-distance spread of both bacterial species. In this phase, endophytic bacterial cells are released either by guttation droplets or through infected stomata and spread via splashing water and wind-driven rain. Xv and Xe seed contamination/infection can occur via two pathways: penetration through fruit lentils (Bashan *et al.*, 1982a) and/or floral structures (Dutta *et al.*, 2016). For both Xv and Xe, seed contamination mainly occurs on the seed surface from infected pulp rather than as internal seed infections (EPPO, 2013). However, neither the transmission of such bacteria from diseased plants to seeds under field conditions nor the correlation between disease quantity and the contamination level of bacteria on/in seeds have been explored to date. This extensive study was performed in confined experimental fields approved by local phytosanitary authorities and located in northern Italy (Emilia Romagna Region) and Serbia (Vojvodina Province), two regions where tomato and pepper represent important crops and where bacterial diseases may cause significant economic losses. The objectives of this study were: (i) to evaluate and quantify disease outbreaks under field conditions, (ii) to assess disease progression and the correlation between disease quantity and the phytosanitary quality of seeds, and (iii) to investigate the relationship between the assessed seed contamination level and the risks of possible disease outbreaks in the following year's crop. These data will be useful for seed companies for the production of seeds with an acceptable phytosanitary quality when disease symptoms are expressed during a vegetative season. The outcome of this study may also aid in the identification of some additional aspects of Xv and Xe plant-to-seed and seed-to-plant transmission in tomato and pepper, respectively, thus shedding light on the epidemiology of these diseases.

Materials and methods

Bacterial strains and plant material

The virulent strains IPV-BO 2684 and KFB29 of Xv and MI-A-6 of Xe, belonging to the bacterial collections of the Department of Agricultural Sciences (University of Bologna, Italy) and the Institute of Field and Vegetable Crops (Novi Sad, Serbia), respectively, were used for this study. To confirm their pathogenicity prior to experiments, Xv and Xe were inoculated and reisolated from fruits and leaves with symptoms taken from tomato and pepper, respectively. All strains were routinely grown on glucose-yeast extract-calcium carbonate agar (GYCA) (Dye, 1962) at 27 ± 1 °C for 48–72 h.

Tomato and pepper cultivars were selected for their high susceptibility to bacterial diseases and adaptation in their respective countries. In Italy, processing tomato cv. VF10 was used,

whereas in Serbia, fresh market tomato cv. Jabučar and bell pepper cv. Amphora were used. Certified tomato and pepper seeds were kindly provided by commercial sources: processing tomato seeds were provided by ISI Sementi s.p.a. (Fidenza, Italy), and fresh market tomato and bell pepper seed were provided by NS-SEME (Novi Sad, Serbia).

Field experiments

The respective plant health authorities were notified of the field experiments, which were conducted in confined experimental fields under the supervision of the local Phytosanitary Service (Cadriano Experimental Station, Bologna, Italy; IFVCS Experimental Station, Novi Sad, Serbia).

Field experiments were performed in Italy on processing tomato–Xv and in Serbia on fresh market tomato–Xv and pepper–Xe pathosystems. The experiments started in 2013 and were conducted during two cropping seasons.

In the first cropping year, for each pathosystem, three plots of 96 plants each (12 plants in each of eight rows) were set. Tomato and pepper seedlings were planted according to the common agricultural procedures followed by farmers for commercial purposes. For processing tomato, each plot consisted of eight rows of 12 plants spaced 0.3 m apart with 0.7 m between rows. For fresh market tomato and bell pepper, each plot consisted of eight rows of 12 plants spaced 0.5 m apart with 1.1 m between rows. Rows of maize were grown between plots to avoid cross-contamination. One, five and 14 plants of each plot in both Italy and Serbia were arbitrarily selected, labelled and experimentally inoculated to obtain approximately 1%, 5% and 15% of infected plants equitably distributed among the plots (Fig. S1). The plants were experimentally inoculated 5 weeks after transplanting. At this point, the phenological growth stage of the tomato and pepper plants was the beginning of flowering and intensive leaf growth and bud shooting, respectively. Late in the afternoon, the labelled plants were spray-inoculated until run-off with a water suspension containing each pathogen at a concentration of approximately 10^8 CFU mL^{-1} . Specifically, Xv and Xe strains were grown on GYCA for 48 h at 28 °C, and the concentration of bacterial cells was determined using a spectrophotometer (Spectronic 20, Bausch and Lomb; optical density at 600 nm = 0.3; $\approx 10^8$ CFU mL^{-1}). After spraying, each inoculated plant was sealed in a polyethylene (PE) bag to maintain high humidity and facilitate pathogen penetration mainly through stomata and hydathodes into the host. Early the following morning, the PE bags were removed. A successful inoculation was demonstrated by symptom development, sampling of leaves with symptoms, and analysis to confirm infection by the respective pathogens. Specifically, to confirm pathogen identity between the inoculum source and reisolated strains, rep-PCR using the primer BOXA1R (5'-CTACGGCAAGGCGACGCT GACG-3') was performed according to Versalovic *et al.* (1994). The plants were cultivated according to the local best agricultural practices. In Italy (processing tomatoes), two harvests were manually performed 7 days apart; in Serbia, fresh market tomatoes and bell peppers were harvested weekly for a period of 2 months (12 harvests in total).

In the second cropping season, the tomato and pepper seeds, generated during the previous year from each experimentally infected field plot, were sown in blotters. The seedlings (at the 3–4-leaf stage) were then transplanted in new experimental fields, which were designed as in the previous year; seedlings generated from commercially certified seeds were also transplanted in an additional plot of 96 plants as a negative control. Rows of maize

were grown between plots to avoid cross-contamination. Again, the plants were cultivated according to the local best agricultural practices. Fruits were harvested, and seeds were extracted according to the procedures described below. Agroclimatic parameters were monitored throughout both growing seasons.

Phytopathometric evaluations

During the first cropping year, phytopathometric assessments in experimental fields were performed weekly, starting from the first appearance of symptoms until harvest. During these assessments, the increase in disease incidence and severity was recorded. Diseased plants were counted (incidence), and each one was assigned to a disease severity class. Disease severity in tomato and pepper affected by *Xanthomonas* spp. strains was evaluated according to five phytopathometric classes, ranging from 0 to 4 (0 = no symptoms; 1 = 1–10 spots on up to 3 leaves; 2 = 11–30 spots on 4–10 leaves; 3 = >30 spots and some confluent necrosis on 5–20 leaves; 4 = confluent necrosis on >20 leaves or branch desiccation) (Giovanardi *et al.*, 2015). Disease scores were calculated as Σ of Q, where Q represents the combination of the disease severity and the incidence (severity \times incidence) at each assessment, as proposed by Garvin & Blankenheim (2013) and implemented by Mehl (2015) for the bacterial leaf blight of maize. The area under the disease progression curve (AUDPC; Van der Plank, 1963) was then calculated according to Madden *et al.* (2007) from the first phytopathometric evaluation to the last assessment before harvest. Moreover, the AUDPCs were statistically evaluated by ANOVA ($\alpha = 0.05$) using SPSS v. 15.0 for Windows.

Seed extraction

Tomato and pepper seeds were produced according to common commercial procedures (Opeña *et al.*, 2001). For both tomato cultivars, seeds were extracted according to the fermentation technique as follows: harvested tomatoes were left in a dark store at 23 ± 1 °C for 2 weeks for post-harvest full maturation before seed extraction. Then, seeds were manually extracted from the pulp, and the mixture of seeds and placental tissue was maintained at 25 ± 1 °C for 24 h. The seeds were then separated from the fermentation mixture, thoroughly washed under running tap water and dried overnight in the dark at 25 ± 1 °C on sterilized paper trays. For bell pepper, seeds were manually collected after pericarp removal, thoroughly washed under running tap water and subsequently dried for 48 h in the dark at 25 ± 1 °C. The seeds produced were weighed and counted for each plot. Finally, all dried seeds were stored in paper boxes and kept in the dark at 4 ± 1 °C.

Seed analysis and contamination rate of seed plots

For each seed lot generated from the experimental plots, seed analysis was performed by dilution plating and molecular assays according to the EPPO (2013) standard diagnostic protocol for *Xanthomonas* spp. causing bacterial spot of tomato and pepper. Seed samples ($n = 10\,000$) from each field plot were soaked in a stomacher bag with a ratio of 4 mL sterile PBS-T ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 19.57 g L⁻¹, KH_2PO_4 1.65 g L⁻¹, Tween 20 0.5 g L⁻¹; pH 7.4) per g of seeds for 14 h at 4 ± 1 °C (EPPO, 2013). The seeds, contained in stomacher bags, were then crushed with a hammer for 2 min, and the maceration fluid was centrifuged at 10 000 g for 20 min at 4 ± 1 °C. The resulting

pellet was then resuspended in 1 mL sterile PBS-T. Each seed extract was divided into two aliquots of 900 and 100 μL . The 900- μL aliquots were used for DNA extraction and purification with the DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions.

Two microlitres of purified DNA was then subjected to a conventional PCR assay using the specific primer sets for the detection of Xv (Bs-XvF: 5'-CCATGTGCCGTTGAAATACTTG -3'; Bs-XvR: 5'-ACAAGAGATGTTGCTATGATTTGC-3') and Xe (Bs-XeF: 5'-CATGAAGAAGCTCGGCGTATCG-3'; Bs-XeR: 5'-GTCCGACATAGTGGACACATAC- 3') (Koenraad *et al.*, 2009) according to EPPO (2013). Expected amplicon sizes were 138 and 173 bp for the Xv-specific and Xe-specific primer sets, respectively. For each sample, the 100- μL aliquot was diluted ten-fold and streaked onto GYCA medium. The tomato and pepper seed extracts were also streaked onto mTMB (McGuire *et al.*, 1986), a specific semiselective medium for Xv/Xe. After incubating for 72–96 h at 28 °C, putative Xv and Xe colonies were selected for purification on GYCA and further identification of axenic colonies was carried out with specific primer sets, as previously described. To confirm pathogen identity between the inoculum source and reisolated strains, rep-PCR with the BOXA1R primer set was performed (Versalovic *et al.*, 1994). Seed samples were assayed in triplicate for each contaminated plot.

The contamination level (CFU) of each seed sample ($n = 10\,000$) was preliminarily calculated as the mean number of colonies of the four ten-fold dilutions (i.e. 1:1; 1:10; 1:100 and 1:1000). Considering the weight per thousand grains (WTG), each seed contamination level was expressed as CFU g⁻¹. The three seed sample ($n = 10\,000$) replicates of each plot were used to calculate the mean contamination level expressed as CFU g⁻¹.

Thereafter, to assess the contamination rates of seeds from each infected field plot (1%, 5% and 15%), 50 replicates of 100 seeds from each plot were analysed by conventional PCR. From each plot, the contamination rates of seeds were calculated according to the formula $p = 1 - (Y/N)^{1/n}$, where N is the number of analysed replicates, n the number of seeds in a replicate and Y the number of healthy groups (Maury *et al.*, 1985; Darasse *et al.*, 2007). Each 100-seed sample was soaked in a stomacher bag in 3 mL sterile PBS-T for 14 h at 4 ± 1 °C (EPPO, 2013). The seed samples were then processed and analysed by conventional PCR with specific primer sets for the detection of Xv and Xe, as described above.

Seed germination rate assay

Seeds generated from both the first and second cropping season in Italy and Serbia were tested to determine their germination rate; *in vitro* germination was carried out according to International Rules for Seed Testing standards (ISTA, 2009). One hundred seeds for each pathosystem were placed on Whatman no. 5 filter paper (Sigma-Aldrich) in Petri dishes and dampened with 5 mL sterile distilled water (SDW); the plates were then placed at 25 ± 1 °C in the dark. Germinated seedlings were counted every day for 14 days. The *in vitro* germination assays were repeated four times for each seed sample, and the results were collected and statistically evaluated by ANOVA ($\alpha = 0.05$) using SPSS v. 15.0 software for Windows.

Seedling grow-out (SGO) assay

To test the seed transmission of Xv and Xe, a SGO assay was performed on blotters by sowing seed samples ($n = 1000$) into pots containing peat. The seedlings were then kept in a climatic

chamber for 4 weeks at 28 ± 1 °C, with a relative humidity (RH) of up to 90%. After 28 days, the seedlings were inspected for typical Xv or Xe symptoms. After 4 weeks, all seedlings were collected and segments of approx. 2 cm were cut from each stem, and pooled in stomacher bags with 30 mL sterile PBS-T. The samples were then crushed by hammering and incubated at room temperature for 30 min. The stem macerates were centrifuged at 10 000 g for 20 min at 4 ± 1 °C and the resulting pellets were resuspended in 1 mL PBS-T. Each sample was analysed by dilution plating and molecular assays, as previously described. Seed samples from each contaminated plot were tested in triplicate.

Results

Field experiments

In the first cropping season in Italy, the first symptoms were detected on processing tomato leaves 2 weeks after inoculation with Xv. These symptoms were observed as necrotic spots and marginal necrotic areas from which the Xv strain IPV-BO 2684 was reisolated. Strain identity was confirmed by rep-PCR assay using the BOXA1R primer set. At harvesting time, bacterial spot was approximately 12%, 60% and 85% in the plots with an initial infection percentage of 1%, 5% and 15%, respectively. Disease severity, calculated according to a disease index scale and assessed for each plant, progressively increased from one observation to the next; therefore, the disease quantity (Q) constantly increased until the last assessment. The increase in the disease progression curve for Xv in infected plots directly correlated with the percentage of initial infection (Fig. 1). The AUDPC value of processing tomato plants inoculated with Xv in the field plot with a 1% initial infection rate was 249, which was approximately six- and ten-fold lower than the field plots with an initial infection rate of 5% (AUDPC = 1512) and 15% (AUDPC = 2654), respectively (Table 1). Xv infections produced spots on a limited number of fruits.

During the second cropping season, no bacterial spot symptoms were recorded in any of the plots where processing tomato plants were obtained from seeds produced during the previous year in diseased plots.

In Serbia, during the first cropping season, leaf spot symptoms on fresh market tomato and bell pepper appeared 2 weeks after the experimental inoculation and increased until the last assessment, which was 18 weeks after transplanting. Symptoms developed on both leaves and fruits. In addition, both Xv KFB29 and Xe MI-A-6 were reisolated from plant tissues with symptoms, and their identity was confirmed by rep-PCR assay using the BOXA1R primer set. Disease quantity (Q) increased in all infected fresh market tomato plots (Fig. 2) until 100% of the plants were diseased (data not shown). Similar results were observed for bell pepper: disease quantity (Q) increased in all plots (Fig. 3) until it reached 100% (data not shown). The AUDPC value of Xv-infected tomato plants in the field with a 1% initial infection rate was 8589, approximately two times lower than that of the fields with 5% (AUDPC = 15 074) and 15% (AUDPC = 18 788) initial infection rates.

For the bell pepper–Xe pathosystem, the calculated AUDPC of the field plot with an initial infection rate of 1% was 5743, and although this was approximately 1.5 times lower than that of the field plot with a 5% initial infection rate (AUDPC = 8522) and almost 3 times lower than in the plot with a 15% initial infection rate (AUDPC = 13 632), AUDPC values did not show significant differences among the different plots (Table 1).

During the second cropping season, no symptoms were recorded in any of the fresh market tomato and bell pepper plots generated with seeds produced in the previous year from diseased plots.

The environmental conditions of the two cropping seasons are reported in the supplementary material (Figs S2 and S3). In Italy, during both cropping seasons, the minimum, maximum and mean temperatures were average for that period, whereas the RH was below average, with values <70% from 20 May to 31 August in 2013 and from 1 July to 31 August in 2015. In Serbia, during both cropping seasons, the minimum, maximum and mean temperatures were average for that period, whereas the RH was below average, with values <80% from 15 July to 26 August in 2013 and from 1 July to 19 August in 2015.

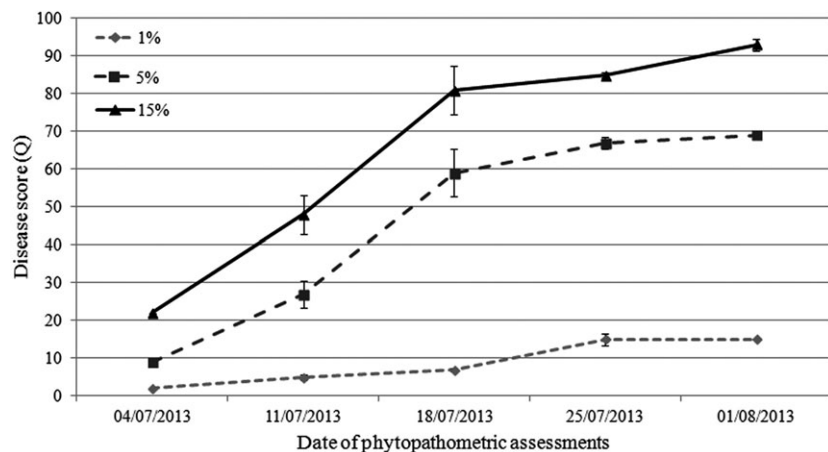


Figure 1 Disease progression curves of bacterial leaf spot caused by *Xanthomonas vesicatoria* on processing tomato plants (cv. VF10) from the beginning of July to 1 August during the first year of experiments in Italy. Bars represent standard deviations at each phytopathometric assessment. The percentage values in the legend represent the initial percentage of inoculated plants in each plot of the experimental field.

Table 1 AUDPC values of field plots calculated for the different pathosystems in Italy and Serbia during the first cropping season.

| Pathosystem | Initial percentage of infected plants | | |
|--|---------------------------------------|-----------|----------|
| | 1% | 5% | 15% |
| <i>Xanthomonas vesicatoria</i> – processing tomato | 249 a | 1512 b | 2654 b |
| <i>X. vesicatoria</i> – fresh market tomato | 8589 a | 15 074 ab | 18 788 b |
| <i>X. euvesicatoria</i> – bell pepper | 5743 a | 8522 a | 13 632 a |

Different letters indicate different statistical classes (Duncan's test, $\alpha = 0.05$).

Harvest and seed production

In Italy, *c.* 65 000 seeds per plot were extracted from processing tomato fruits of Xv-infected fields in the first cropping season, and *c.* 44 000 seeds per plot in the second cropping season. From the negative control field plot, *c.* 37 000 seeds were extracted.

In Serbia, *c.* 60 000 seeds per plot from fresh market tomato and *c.* 40 000 seeds per plot from bell pepper were extracted in the first cropping season, and *c.* 50 000 and *c.* 74 000 seeds from tomato and pepper, respectively, in the second cropping season. From the negative control field plots, *c.* 57 000 of fresh market tomato seeds and *c.* 39 000 of bell pepper seeds were extracted.

The WTG was 2.61, 2.60 and 6.96 g for processing tomato, fresh market tomato and bell pepper seeds, respectively.

Seed analysis and contamination rate of seed lots

Tomato and pepper seed lots produced during both cropping seasons were tested according to the EPP0 (2013) standard diagnostic protocol for *Xanthomonas* spp. (Table 2). For seeds produced during the first cropping season, Xv was not detected by either PCR or direct isolation on GYCA and mTMB media from tomato seeds

produced in Italy and in Serbia. For pepper, Xe was consistently detected by PCR and recovered on mTMB and GYCA media from all seed lots produced in the different field plots. The seed contamination level was assessed as 34 (SD = 13), 37 (SD = 22) and 100 (SD = 48) CFU g⁻¹ in the 1%, 5% and 15% infected plots, respectively. For seeds produced during the second cropping season, direct isolation and molecular analysis did not result in the detection of Xv and Xe in any plot in Italy or Serbia.

The contamination rate of tomato seed lots produced during the first cropping season in Italy and Serbia, as determined according Maury *et al.* (1985), was negative for both processing and fresh market tomato produced in Xv-infected plots. For pepper seeds, the seed contamination rate was 1.50%, 3.17% and 3.17% in the plots infected with Xe at 1%, 5% and 15%, respectively (Table 2). The seed contamination rate of all seed lots produced during the second cropping season in Italy and Serbia tested negative for Xv and Xe.

Seed germination rate assay

All seed lots produced during both cropping seasons in Italy and Serbia were subjected to quality testing by assessing their germination rate and their ability to produce marketable seedlings. The germination rate of tomato and pepper seeds produced during the first cropping season was *c.* 95–98%, with no significant differences ($P \geq 0.05$) among seed lots produced in plots with different disease quantities. Similar results were obtained from seed lots produced during the second cropping year. No significant differences ($P \geq 0.05$) in the germination rate (ranging from 90% to 98%) were observed among the different seed lots produced and the uninfected plots (negative controls).

SGO assay

The assessment of disease incidence during the SGO assays did not reveal any disease symptom on seedlings obtained from seeds produced in both the first and second cropping years in Italy and Serbia. Additionally,

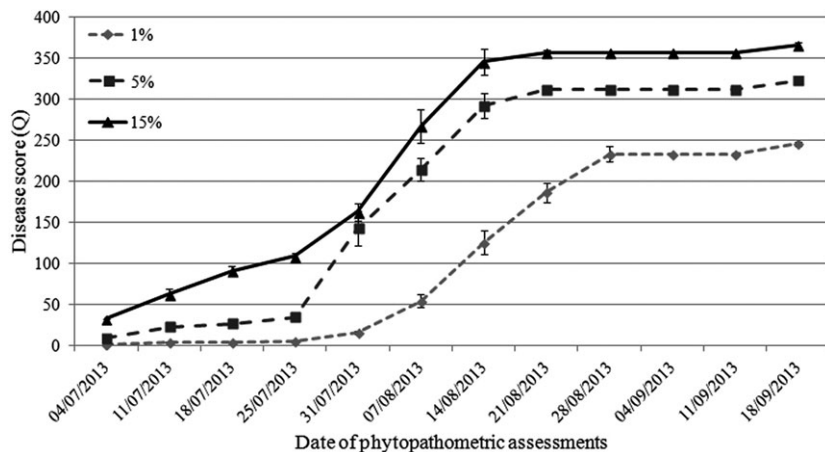


Figure 2 Disease progression curves of bacterial leaf spot caused by *Xanthomonas vesicatoria* on fresh market tomato plants (cv. Jabučar) from the beginning of July to the second week of September during the first year of experiments in Serbia. Bars represent standard deviations at each phytopathometric assessment. The percentage values in the legend represent the initial percentage of inoculated plants in each plot of the experimental field.

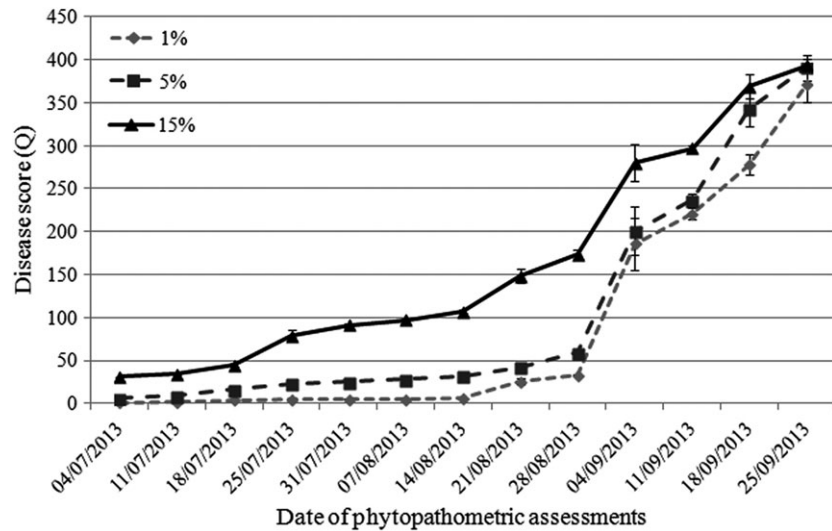


Figure 3 Disease progression curves of bacterial leaf spot caused by *Xanthomonas euvesicatoria* on bell pepper plants (cv. Amphora) from the beginning of July to the end of September during the first year of experiments in Serbia. Bars represent standard deviations at each phytopathometric assessment. The percentage values in the legend represent the initial percentage of inoculated plants in each plot of the experimental field.

microbiological and molecular analysis performed on each seedling macerates and their DNA extracts were all negative for the presence of Xv and Xe.

Discussion

Xv and Xe are phytopathogenic bacteria of great concern to seed companies and farmers. Xv has been recorded in several important seed-producing countries (e.g. Brazil, Mexico, USA, India, Thailand), whereas Xe exhibits a far more restricted distribution (EFSA Panel on Plant

Health, 2014). Both Xv and Xe are seedborne pathogens, and infested seeds serve as a main source of primary inoculum in transplant and fruit production systems (Jones *et al.*, 1993; Leite *et al.*, 1995; Dutta *et al.*, 2014).

Seed companies are devoted to producing tomato and pepper seeds under pathogen exclusion conditions, either in areas where the pathogens have never been reported, or under strict hygiene practices (Gitaitis & Walcott, 2007). Nonetheless, disease symptoms may appear in seed production fields due to the use of seeds that are contaminated at a level under the pathogen detection threshold or because the production area is not sufficiently surveyed. It remains unknown whether a consistent positive correlation exists between seed contamination rate and disease outbreak and whether a similar correlation exists between disease quantity, as measured in fields devoted to seed production, and the phytosanitary quality of the resulting seeds.

In the present study devoted to simulate bacterial spot outbreaks occurring from external sources of inoculum, the results obtained during the first cropping season revealed a positive correlation between the incidence of initial infection, disease progression and disease quantity/score (Q) for both Xv and Xe, as confirmed by the calculated AUDPC values. The disease scores measured on tomato were remarkably different between the Italian and Serbian fields; these differences might be explained by the length of the growing cycle, which is considerably longer for fresh market tomato (7 weeks longer) than that for processing tomato, and by the presence of more favourable environmental conditions for disease outbreak in Serbia than in Italy (e.g. higher RH).

Similar disease progression occurred in bell pepper, because monitoring and harvesting of peppers continued for an additional 8 weeks after processing tomato harvest. In addition, even though AUDPC values from 1% infected plots appeared lower than those from 5% to 15% infected plots, the differences were not significant.

Table 2 Contamination levels and rates of *Xanthomonas vesicatoria* (Xv) and *Xanthomonas euvesicatoria* (Xe) in tomato and pepper seeds, respectively, produced in Italy and Serbia during the first cropping season from field plots experimentally infected at different initial contamination incidences.

| Pathosystem | Seed contamination level (CFU g ⁻¹) | Contamination rate (%) |
|------------------------------|---|------------------------|
| Italy | | |
| Xv 1% – processing tomato | 0 | 0 |
| Xv 5% – processing tomato | 0 | 0 |
| Xv 15% – processing tomato | 0 | 0 |
| Serbia | | |
| Xv 1% – fresh market tomato | 0 | 0 |
| Xv 5% – fresh market tomato | 0 | 0 |
| Xv 15% – fresh market tomato | 0 | 0 |
| Serbia | | |
| Xe 1% – bell pepper | 34 (SD = 13) | 1.50 |
| Xe 5% – bell pepper | 37 (SD = 22) | 3.17 |
| Xe 15% – bell pepper | 100 (SD = 48) | 3.17 |

The bacterial colonies (CFU) are related to 1 g of seeds. To determine the seed contamination rate of each seed lot, 50 replicates of 100 seeds were tested. The contamination rate (p) was calculated according to the formula $p = 1 - (YN)^{1/n}$, where N is the number of analysed replicates, n is the number of seeds in a replicate and Y is the number of noncontaminated subsamples (Maury *et al.*, 1985). SD, standard deviation.

This could be due to the length of the growing cycle, but also to the more efficient spread of Xe on bell pepper, in comparison to that of Xv in fresh market tomato in the same environmental conditions.

In Italy, Xv infections produced spots on a limited number of fruits; this might be explained by the low daily mean RH (<60%), which was consistently measured at anthesis and fruit development and ripening (July–August 2013). Conversely, during the same physiological growth stages in Serbia, a daily mean RH > 80% was consistently recorded. Therefore, the RH conditions, which were more favourable for bacterial infection than those recorded in Italy, might explain the high disease incidence and severity expression on both fresh market tomato and bell pepper. Given that no outside sources of inoculum were present during the field experiments, bacterial disease development in the plots was the exclusive result of infection from the artificial inoculum sources, as previously discussed by Kocks & Zadoks (1996) for other xanthomonads. In all inoculated plots, typical foci appeared exclusively around the experimentally inoculated plants, which therefore served as inoculum sources. As already described by Zadoks & Van den Bosch (1994) in their theory of foci, in the present experiments the disease expanded radially from the inoculated plants, apparently approaching a constant rate of expansion. The development of distinct foci in tomato fields and their spatial parameters will surely deserve more attention in the future in order to implement effective management techniques. Finally, the pathogens were reisolated from infected plants, and their identities were confirmed by rep-PCR as the experimental inoculum sources (i.e. strains IPV-BO 2684 and KFB29 of Xv and MI-A-6 of Xe).

Despite the positive correlation observed between the incidence of initial infection, disease progression and AUDPC values for both Xv and Xe, the analysis of seeds produced during the first cropping season did not exhibit any apparent correlation between disease quantity over time (AUDPC), as measured in the production fields, and contamination rates of seeds produced for either the tomato or pepper pathosystems. In the case of tomato, none of the seed analysis detected Xv, although 58% and 63% of the total seeds produced in Italy and in Serbia were tested, respectively, and the disease observed in the field plots was remarkably severe and present on all aerial parts, i.e. leaves, fruits, petioles and stems. Conversely, seed contamination rates were between 1.50% and 3.17% for pepper seed lots produced in diseased plots. These pepper seed lots tested positive for Xe detection by conventional PCR and by isolation on semiselective media. These results confirm seed contamination by Xe; however, the infection was characterized by relatively low rates and non-uniform distribution of Xe among the seed lots. Pathogen reisolation indicated that the seed population density for Xe was *c.* 34–100 CFU g⁻¹.

The seed extraction protocol based on the fermentation of tomato pulp (Opeña *et al.*, 2001) is thought to play a major role in the viability and detectability of infecting bacteria. This method is able to decrease the

viability of bacterial populations contaminating the seeds, leading to a consistent reduction in the density of viable bacterial cells on tomato seed surfaces (Chambers & Merriman, 1975). Bacteria in the viable but nonculturable (VBNC) state, which fail to grow on the routine bacteriological media, have been described (Oliver, 2005). These bacteria are in a state of very low metabolic activity and do not divide. This feature occurs in response to stress (e.g. due to adverse nutrient, temperature, osmotic, oxygen, and light conditions; Stokell & Steck, 2012). In the present case, seeds were analysed immediately after their production, and they were stored according to the best possible procedures (i.e. dry seeds stored at 4 °C in the dark). Therefore, it is presumed that no stress or other adverse conditions could have caused the development of a VBNC status in both Xv and Xe. Additional confirmation of this conclusion was obtained by molecular assays. These assays could detect the DNA present in VBNC, but the results were negative for the presence of the pathogen. When the pathogen was reisolated on semiselective medium, PCR consistently detected its presence in DNA extracts.

Throughout the second cropping season, no bacterial spot symptoms were observed in pepper or tomato plants. Moreover, in seeds extracted in the second cropping season, the pathogens were not detected by any of three biologically different tests: microbiological, molecular and SGO assays. Therefore, the lack of symptom development in all field plots indicates that seeds were not infected or contaminated by Xv or that the pathogen transmission was negligible due to a low bacterial load of Xe on pepper seeds, which was not sufficient to cause disease development in the next cropping season. Because Bashan *et al.* (1982b) observed that Xv can survive in tomato seeds for a long time, up to 8 years, it may be presumed that the viability of the pathogen in seeds used in the following cropping season was not dissimilar to the contamination level assessed after seed production.

Inoculum transmission thresholds in seeds have been studied in some pathosystems in order to identify the correlation between seed infection rates and disease outbreaks, as in the case of *Pseudomonas savastanoi* pv. *phaseolicola* in bean seeds (Taylor *et al.*, 1979) or *Xanthomonas campestris* pv. *campestris* in crucifer seeds (Schaad *et al.*, 1980). In these studies, the inoculum thresholds were set either arbitrarily (e.g. using experimental seed inoculation) or simply with field observations. In the case of tomato and pepper seeds, the correlation between inoculum thresholds of Xv and Xe and disease outbreaks has not been previously evaluated under field conditions. Additionally, to the best of the authors' knowledge, no studies in the literature have elucidated the quantitative/qualitative correlation between disease quantity observed in tomato/pepper fields, the concentration of inoculum in the seeds produced in those fields and the bacterial spot outbreak risks posed by the seeds on the next crop.

This is the first report on the evaluation of a seed contamination threshold for bacterial spot in pepper. The

results from this study showed that a seed contamination level higher than 100 CFU g⁻¹ is needed for a disease outbreak. Due to the highly polycyclic nature of the disease, it is important to emphasize that the threshold level may be variable, considering that pepper-growing areas have quite different climatic conditions and/or different agronomic practices (e.g. higher seeding rates).

Conversely, for the Xv–tomato pathosystem, the data showed a total lack of inoculum transmission, confirmed by the absence of living cells on semiselective media used for reisolation. Xv, during SGO assays and under field conditions, was not transmitted either to seedlings or to seeds in either Italy or Serbia. The results obtained from molecular and microbiological assays on seed extracts of processing tomatoes also suggest the important role of extraction protocols in seed sanitation (i.e. a fermentation step). The fermenting process could be considered an appropriate seed surface disinfection step that does not affect seed quality, as confirmed by *in vitro* germination results. The results obtained from SGO assays, in particular those related to the Xe–pepper pathosystem, for which plant-to-seed transmission was successfully achieved, suggest that the bacterial loads in contaminated seeds were not sufficient to develop symptoms, even using optimal controlled conditions for both host and pathogen. As observed in the field, the pathogen population on seeds of the two strains used were confirmed to be insufficient for disease outbreak.

This study provides new information on the seed transmission of bacterial spot as well as a deeper knowledge of epidemics in the field. This data will facilitate a better understanding of the epidemiology of *Xanthomonas* spp. seedborne bacteria.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Design of experimental field plots used during the first cropping season in Italy and Serbia. This design was used to evaluate and quantify disease outbreaks under field conditions and assess disease progression from different initial infection percentage of approx. 1%, 5% and 15% by *Xanthomonas vesicatoria* in processing and fresh market tomato and *Xanthomonas euvesicatoria* in bell pepper. The position of each experimentally inoculated plant is highlighted by a cross.

Figure S2. Meteorological data reported in Italy (a) and Serbia (b) during the first cropping season in 2013 from 1 April to the last harvest.

Figure S3. Meteorological data reported in Italy (a) and Serbia (b) during the second cropping season in 2015 from 1 April to last harvest.