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

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3 D. Giovanardi [1], E. Biondi [2], M. Ignjatov [3], R. Jevtić [3], E. Stefani [1].

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5 [1]Department of Life Sciences, University of Modena and Reggio Emilia, via Amendola 2, 42122

6 Reggio Emilia, Italy. [2]Department of Agricultural Sciences, University of Bologna, viale Fanin 44,

7 40127 Bologna, Italy.[3]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi

8 Sad, Serbia.

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10 E-mail address: davide.giovanardi@unimore.it

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12 **ABSTRACT**

13 The impact of disease outbreaks on the phytosanitary quality of seeds was investigated for the

14 following pathosystems: tomato-*Xanthomonas vesicatoria* and pepper-*Xanthomonas euvesicatoria.*

15 This study, which was performed in Italy and in Serbia, aimed to evaluate the season-to-season

16 transmission of phytopathogenic regulated bacteria associated with phytosanitary risks posed by

17 seeds produced in areas where bacterial infections are possible. For each pathosystem, field plots

18 were experimentally inoculated to simulate an initial infection rate of 1%, 5% and 15%. The area

19 under the disease progress curve (AUDPC) was calculated for each field plot, the produced seeds

20 were analysed to determine the contamination level and rate, and the plant-to-seed transmission was

21 evaluated by a seedling grow-out (SGO) assay. To investigate transmission under field conditions, a

22 second-year experiment was performed, wherein seeds collected from the first year were used to

23 establish new field plots. During the first growing season, AUDPC values were positively

24 correlated with the percentages of initial infection for each pathosystem. Seed contamination levels

25 ranged from 34 to 100 CFU g^{-1}, and the contamination rate ranged from 1.50% up to 3.17% for

26 *Xanthomonas euvesicatoria*, whereas processing and fresh market tomato seeds produced both in

27 Italy and Serbia were not infected by *Xanthomonas vesicatoria*. During SGO assays and the second
28 cropping year, no symptoms were observed in either tomato or pepper plants. Therefore, the
29 calculated pepper seed contamination rate for *Xanthomonas euvesicatoria* appeared to be less than
30 the threshold necessary to initiate a disease outbreak. Finally, all seeds obtained during the second
31 cropping year were uninfected.

32

33 **Keywords**: Seed-borne bacteria, bacterial leaf spot, pathogen transmission, phytosanitary seed
34 quality.

35

36 **INTRODUCTION**

37 Infected seeds are the most important pathway for the introduction and spread of several plant
38 pathogenic bacteria that may affect both fruit quality and plant viability, thus causing economic
39 losses worldwide (Gitaitis & Walcott, 2007). Among other bacterial diseases, tomato can be
40 affected by bacterial spot caused by *Xanthomonas vesicatoria* (*e.g.,* Doidge, 1920; Vauterin *et al.*
41 1995) (Xv) and *X. euvesicatoria* (Jones *et al.* 2004) (Xe). The latter is particularly aggressive
42 against pepper (Ignjatov *et al.* 2010). These bacteria are spread primarily through
43 contaminated/infected seeds as the primary source of *inoculum* (Dutta *et al.* 2014) and are listed as
44 quarantine organisms by the European Plant Protection Organization (EPPO). These pathogens can
45 survive in seeds for extended periods (Bashan *et al.* 1982a); such survival ability allows them long-
46 distance dissemination and facilitates their introduction into pathogen-free areas through seed trade.
47 Seed testing and certification (EPPO, 2013) and seed production in pathogen-free areas with no
48 conducive environmental conditions are the most important management strategies for the
49 preventive control of these pathogens. Sanitation of potentially contaminated seeds is only partially
50 achieved using common disinfection methods, *e.g.,* heat treatment, fermentation of fruit pulp as in
51 the case of tomato (Chambers & Merriman, 1975; Dhanvantari, 1989), or chlorine/acid treatment
52 for pepper seeds (Dempsey & Walker, 1973). So far, there is no method available that can ensure

53 the complete eradication of pathogens from naturally infected seeds without dramatically reducing
54 seed germination (Dhanvantari, 1989). Despite the use of phytosanitary certification and quarantine
55 procedures in domestic and international seed trade, which can considerably reduce disease
56 incidence, severe epidemics are occasionally reported (Gitaitis & Walcott, 2007).
57 Transplant production studies on the transmissibility of *Clavibacter michiganensis* subsp.
58 *michiganensis* (Smith) (Davis *et al.* 1984; Strider, 1969), the causal agent of tomato bacterial
59 canker, revealed that a single infected tomato seed among 10,000 is sufficient to initiate an
60 epidemic under favourable conditions (Chang *et al.* 1991). Similar studies are currently not
61 available for Xv or for Xe. Very few studies have been conducted under field conditions to evaluate
62 the seed contamination threshold necessary for pathogen transmission from seed to plants. Chang et
63 al. (1991) demonstrated a systemic infection in tomato transplants grown from infected seeds
64 containing approx. 10^4 CFU/g^{-1} of *C. michiganensis* subsp. *michiganensis*. Conversely, knowledge
65 regarding the Xv and Xe seed contamination thresholds needed for disease expression on tomato
66 and pepper plants under field conditions is lacking. Bacterial spot is a polycyclic disease. Secondary
67 *inocula* might be abundantly produced during the cropping period and may play a key role in the
68 short-distance spread of both bacterial species. In this phase, endophytic bacterial cells are released
69 either by guttation droplets or through infected stomata and spread via splashing water and wind-
70 driven rain. Xv and Xe seed contamination/infection can occur via two pathways: penetration
71 through fruit lenticels (Bashan *et al.* 1982a) and/or floral structures (Dutta *et al.* 2016). For both
72 causal agents of bacterial spot of tomato and pepper plants (Xv and Xe), seed contamination mainly
73 occurs on the seed surface from infected pulp rather than as internal seed infections (EPPO, 2013).
74 However, neither the transmission of such bacteria from diseased plants to seeds under field
75 conditions nor the correlation between disease quantity, and the contamination level of bacteria
76 on/in seeds have been explored to date. This extensive study was performed in confined
77 experimental fields approved by local phytosanitary authorities and located in Northern Italy
78 (Emilia Romagna Region) and Serbia (Vojvodina Province), two regions where tomato and pepper

79 represent important crops and where bacterial diseases may cause significant economic losses. The
80 objectives of this study were (i) to evaluate and quantify disease outbreaks under field conditions,
81 (ii) to assess disease progression and the correlation between disease quantity and the phytosanitary
82 quality of seeds, and (iii) to investigate the relationship between the assessed seed contamination
83 level and the risks of possible disease outbreaks in the following year's crop. These data will be
84 useful for seed companies for the production of seeds with an acceptable phytosanitary quality
85 when disease symptoms are expressed during a vegetative season. The outcome of this study may
86 also aid in the identification of some additional aspects of Xv and Xe plant-to-seed and seed-to-
87 plant transmission in tomato and pepper, respectively, thus shedding light on the epidemiology of
88 these diseases.
89

90 **MATERIALS AND METHODS**

91 **Bacterial strains and plant material**

92 The virulent strains IPV-BO 2684 and KFB29 of Xv and MI-A-6 of Xe, belonging to the bacterial
93 collections of the Department of Agricultural Sciences (University of Bologna, Italy) and the
94 Institute of Field and Vegetable Crops (Novi Sad, Serbia), respectively, were used for this study. To
95 confirm their pathogenicity prior to experiments, Xv and Xe were inoculated and re-isolated from
96 symptomatic fruits and leaves of tomato and pepper, respectively. All strains were routinely grown
97 on glucose-yeast extract-calcium carbonate medium (GYCA) (Dye, 1962) at 27.0 °C ± 1 °C for 48-
98 72 h.
99 Tomato and pepper cultivars were selected for their high susceptibility to bacterial diseases and
100 adaptation in their respective countries. In Italy, processing tomato cv. VF10 was used, whereas in
101 Serbia, fresh market tomato cv. Jabučar and bell pepper cv. Amphora were used. Certified tomato
102 and pepper seeds were kindly provided by commercial sources: processing tomato seeds were
103 provided by ISI Sementi s.p.a. (Fidenza, Italy), and fresh market tomato and bell pepper seed were
104 provided by NS-SEME (Novi Sad, Serbia).

105

106 **Field experiments**

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

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

113 In the first cropping year, for each pathosystem, three plots of 96 plants each (12 plants in each of 8
114 rows) were set. Tomato and pepper seedlings were planted according to the common agricultural
115 procedures followed by farmers for commercial purposes. For processing tomato, each plot
116 consisted of 8 rows of 12 plants spaced 0.3 m apart with 0.7 m between rows. For fresh market
117 tomato and bell pepper, each plot consisted of 8 rows of 12 plants spaced 0.5 m apart with 1.1 m
118 between rows. Rows of maize were grown between plots to avoid cross contamination. One, five
119 and fourteen plants of each plot in both Italy and Serbia were arbitrarily selected, labelled and
120 experimentally inoculated to obtain approximately 1%, 5% and 15% of infected plants equitably
121 distributed among the plots (see supplementary material S1). The plants were experimentally
122 inoculated five weeks after transplanting. At this point, the phenological growth stage of the tomato
123 and pepper plants was the beginning of flowering and intensive leaf growth and bud shooting,
124 respectively. Late in the afternoon, the labelled plants were spray-inoculated until run-off with a
125 water suspension containing each pathogen at a concentration of approximately 10^8 CFU ml^{-1}.
126 Specifically, Xv and Xe strains were grown on GYC agar for 48 h at 28 °C, and the concentration
127 of bacterial cells was determined using a spectrophotometer (Spectronic 20; Bausch and Lomb,
128 Rochester, NY) (optical density at 600 nm = 0.3; $\approx 1 \times 10^8$ CFU ml^{-1}). After spraying, each
129 inoculated plant was sealed in a polyethylene (PE) bag to facilitate water congestion and pathogen
130 penetration mainly through stomata and hydathodes into the host. The following day, early in the

131 morning, the PE bags were removed. A successful inoculation was demonstrated by symptom
132 development, sampling of symptomatic leaves and analysis to confirm infection by the respective
133 pathogens. Specifically, to confirm pathogen identity between the *inoculum* source and re-isolated
134 strains, rep-PCR using the primer BOX A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) was
135 performed according to Versalovic *et al.* 1994. The plants were cultivated according to the local
136 best agricultural practices. In Italy (processing tomatoes), two harvests were manually performed 7
137 days apart; in Serbia, fresh market tomatoes and bell peppers were weekly harvested for a period of
138 2 months (12 harvests in total).
139 In the second cropping season, the tomato and pepper seeds, generated during the previous year
140 from each experimentally infected field plot, were sown in blotters. The seedlings (at the 3rd-4th leaf
141 stage) were then transplanted in new experimental fields, which were designed as in the previous
142 year; seedlings generated from commercially certified seeds were also transplanted in an additional
143 plot of 96 plants as a negative control. Rows of maize were grown between plots to avoid cross
144 contamination. Again, the plants were cultivated according to the local best agricultural practices.
145 Fruits were harvested, and seeds were extracted according to the procedures described below. Agro-
146 climatic parameters were monitored throughout both growing seasons.
147
148 **Phytopathometric evaluations**
149 During the first cropping year, phytopathometric assessments in experimental fields were weekly
150 performed, starting from the first appearance of symptoms until harvest. During these assessments,
151 the increase in disease incidence and severity was recorded. Therefore, diseased plants were
152 counted (incidence), and each one was assigned to a disease severity class. Disease severity in
153 tomato and pepper affected by *Xanthomonas* spp. strains was evaluated according to five
154 phytopathometric classes, ranging from 0 to 4 (0 = no symptoms; 1 = 1-10 spots on up to 3 leaves; 2
155 = 11-30 spots on 4 to 10 leaves; 3 = more than 30 spots and some confluent necrosis on 5 to 20
156 leaves; 4 = confluent necrosis on more than 20 leaves or branch desiccation) (Giovanardi *et al.*

157 2015). Disease scores were calculated as Σ of Q, where Q represents the combination of the disease
158 severity and the incidence (severity × incidence) at each assessment. The area under the disease
159 progression curve (AUDPC) (Van der Plank, 1963) was then calculated according to Madden *et al.*
160 (2007) from the first phytopathometric evaluation to the last assessment before harvest. Moreover,
161 the AUDPCs were statistically evaluated by ANOVA (p 0.05) using SPSS 15.0 software for
162 Windows®.

163

164 **Seed extraction**

165 Tomato and pepper seeds were produced according to common commercial procedures (Opeña *et*
166 *al.* 2001). For both tomato cultivars, seeds were extracted according to the fermentation technique
167 as follows: harvested tomatoes were left in a dark store at 23.0 °C ± 1 °C for 2 weeks for post-
168 harvest full maturation before seed extraction. Then, seeds were manually extracted from the pulp,
169 and the mixture of seeds and placental tissue was maintained at 25.0 °C ± 1 °C for 24 h. The seeds
170 were then separated from the fermentation mixture, thoroughly washed under running tap water and
171 dried overnight in the dark at 25.0 °C ± 1 °C on sterilized paper trays. For bell pepper, seeds were
172 manually collected after pericarp removal, thoroughly washed under running tap water and
173 subsequently dried for 48 h in the dark at 25.0 ± 1 °C. The seeds produced were weighed and
174 counted for each plot. Finally, all dried seeds were stored in paper boxes and kept in the dark at 4.0
175 °C ± 1 °C.

176

177 **Seed analysis and contamination rate of seed plots**

178 For each seed lot generated from the experimental plots, seed analysis was performed by dilution
179 plating and molecular assays according to the EPPO (2013) standard diagnostic protocol for
180 *Xanthomonas* spp. causing bacterial spot of tomato and pepper. Seed samples (*n = 10,000*) from
181 each field plot were soaked in a Stomacher bag with a ratio of 4 ml of sterilised PBS-T (Na_2HPO_4 •
182 $12H_2O$ 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 4.0 °C ±

183 1 °C (EPPO, 2013). The seeds, contained in Stomacher bags, were then crushed with a hammer for
184 2 min, and the maceration fluid was centrifuged at 10,000 x *g* for 20 min at 4.0 °C ± 1 °C. The
185 resulting pellet was then resuspended in 1 ml of sterilized PBS-T. Each seed extract was divided
186 into two aliquots of 900 and 100 µl. The 900-µl aliquots were used for DNA extraction and
187 purification with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the
188 manufacturer's instructions.
189 Two µl of purified DNA was then subjected to conventional PCR assay using the specific primer
190 sets for the detection of Xv (Bs-XvF: 5′-CCA TGT GCC GTT GAA ATA CTT G -3′; Bs-XvR: 5′-
191 ACA AGA GAT GTT GCT ATG ATT TGC-3′) and Xe (Bs-XeF: 5′-CAT GAA GAA CTC GGC
192 GTA TCG-3′; Bs-XeR: 5′-GTC GGA CAT AGT GGA CAC ATA C- 3′) (Koenraadt *et al.* 2009)
193 according to EPPO (2013). Expected amplicon sizes were 138 bp and 173 bp for the Xv-specific
194 and Xe-specific primer sets, respectively. For each sample, the 100-µl aliquot was ten-fold diluted
195 and streaked onto GYCA medium. The tomato and pepper seed extracts were also streaked onto
196 mTMB (McGuire *et al.* 1986), a specific semi-selective medium for Xv/Xe. After incubating for
197 72-96 h at 28 °C, putative Xv and Xe colonies were selected for purification on GYC agar and
198 further identification of axenic colonies with specific primer sets, as previously described. To
199 confirm pathogen identity between the inoculum source and re-isolated strains, rep-PCR with the
200 BOX A1R primer set was performed (Versalovic *et al.* 1994). Seed samples were assayed in
201 triplicate for each contaminated plot.
202 The contamination level (CFU) of each seed sample ($n = 10{,}000$) was preliminary calculated as the
203 mean number of colonies of the four ten-fold dilutions (*i.e.,* 1:1; 1:10; 1:100 and 1:1000).
204 Considering the weight per thousand of grain (WTG), each seed contamination level was expressed
205 as CFU g^{-1}. The three seed sample ($n = 10{,}000$) replicates of each plot were used to calculate the
206 mean contamination level expressed as CFU g^{-1}.
207 Thereafter, to assess the contamination rates of seeds from each infected field plot (*i.e.,* 1, 5 and
208 15%), 50 replicates of 100 seeds from each plot were analysed by conventional PCR. From each

209 plot, the contamination rates of seeds were calculated according to the formula $p = 1\text{-}(Y/N)^{1/n}$, where
210 N is the number of analysed replicates, n the number of seeds in a replicate and Y the number of
211 healthy groups (Maury *et al.* 1985; Darrasse *et al.* 2007). Each 100-seed sample was soaked in a
212 Stomacher bag in 3 ml of sterilized PBS-T for 14 h at 4.0 °C ± 1 °C (EPPO, 2013). The seed
213 samples were then processed and analysed by conventional PCR with specific primer sets for the
214 detection of Xv and Xe, as described above.
215
216 **Seed germination rate assay**
217 Seeds generated from both first and second cropping season in Italy and Serbia were tested to
218 determine their germination rate; *in vitro* germination was carried out according to International
219 Rules for Seed Testing standards (ISTA, 2009). One hundred seeds for each pathosystem were
220 placed on Whatman No. 5 filter paper (Sigma-Aldrich, Saint Louis, Missouri) in Petri dishes and
221 dampened with 5 ml of sterilized distilled water (SDW); the plates were then placed at 25.0 °C ± 1
222 °C in the dark. Germinated seedlings were counted every day for 14 days. The *in vitro* germination
223 assays were repeated four times for each seed sample, and the results were collected and statistically
224 evaluated by ANOVA (p 0.05) using SPSS 15.0 software for Windows®.
225
226 **Seedling grow-out (SGO) assay**
227 To test the seed transmission of Xv and Xe, a SGO assay was performed on blotters by sowing seed
228 samples ($n = 1,000$) into pots containing peat. The seedlings were then kept in a climatic chamber
229 for 4 weeks at 28.0 °C ± 1 °C, with a relative humidity (RH) of up to 90%. After 28 days, the
230 seedlings were inspected for typical Xv or Xe symptoms. After 4 weeks, all seedlings were
231 collected; then, segments of approx. 2 cm were cut from each stem and pooled in Stomacher bags
232 with 30 ml of sterilized PBS-T. The samples were then crushed by hammering and incubated at
233 room temperature for 30 min. The stem macerates were centrifuged at 10,000 x *g* for 20 min at 4.0
234 °C ± 1 °C and the resulting pellets were resuspended in 1 ml of PBS-T. Each sample was analysed

235 by dilution plating and molecular assays, as previously described. Seed samples from each
236 contaminated plot were tested in triplicate.
237

238 RESULTS

239 Field experiments

240 In the first cropping season in Italy, the first symptoms were detected on processing tomato leaves 2
241 weeks after the inoculation with Xv by surveying the experimental fields. These symptoms were
242 observed as necrotic spots and marginal necrotic areas from which the Xv strain IPV-BO 2684 was
243 re-isolated. Strain identity was confirmed by rep-PCR assay using the BOX A1R primer set. At
244 harvesting time, 14 weeks after transplanting, the final disease incidence calculated for bacterial
245 spot was approx. 12%, 60% and 85% in the plots with an initial infection percentage of 1%, 5% and
246 15%, respectively. Disease severity, calculated according to a disease index scale and assessed for
247 each plant, progressively increased from one observation to the next; therefore, the calculated
248 disease quantity (Q) constantly increased until the last assessment. The increase in the disease
249 progression curve for Xv in infected plots directly correlated with the percentage of initial infection
250 (Figure 1). The AUDPC value of processing tomato plants inoculated with Xv in the field plot with
251 a 1% initial infection rate was 249, which was approx. six- and ten-fold lower than the field plots
252 with an initial infection rate of 5% and 15% (AUDPC, 1512 and 2654, respectively) (Table 1). Xv
253 infections produced spots on a limited number of fruits.
254 During the second cropping season, no bacterial spot symptoms were recorded in any of the plots
255 where processing tomato plants were obtained from seeds produced during the previous year in
256 diseased plots.
257 In Serbia, during the first cropping season, leaf spot symptoms on fresh market tomato and bell
258 pepper appeared 2 weeks after the experimental inoculation and increased until the last assessment,
259 which was 18 weeks after transplanting. Symptoms developed on both leaves and fruits. In addition,
260 both the Xv strain KFB29 and the Xe strain MI-A-6 were re-isolated from symptomatic plant

tissues, and their identity was confirmed by rep-PCR assay using the BOX A1R primer set. Disease quantity (Q) increased in all infected fresh market tomato plots (Figure 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 (Figure 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, approx. two times lower than that of the fields with 5% (AUDPC = 15074) and 15% (AUDPC = 18788) initial infection rates.

For the bell pepper-Xe pathosystem, even though the calculated AUDPC of the field plot with an initial infection rate of 1% was 5743, approx. 1.5 times lower than that of the field plot with a 5% initial infection rate (AUDPC = 8522) and almost 3 times lower than that calculated in the plot with a 15% initial infection rate (AUDPC = 13632), AUDPC values did not show statistical 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 (Figures 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 May 20th to August 31st in 2013 and from July 1st to August 31st in the second cropping season. 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 July 15th to August 26th in 2013 and from July 1st to August 19th in the second cropping season.

Harvest and seed production

In the first cropping season in Italy, *ca.* 65,000 seeds per plot (*ca.* 170 g per plot) were extracted from processing tomato fruits of Xv-infected fields. In the second cropping season, *ca.* 44,000 seeds

286 per plot (*ca.* 114 g per plot) were extracted from the fruits of Xv-infected processing tomato fields.
287 From the negative control field plot, *ca.* 37,000 seeds (*ca.* 95 g) were extracted.
288 In the first cropping season in Serbia, *ca.* 60,000 seeds per plot (*ca.* 156 g per plot) of fresh market
289 tomato and *ca.* 40,000 seeds per plot (*ca.* 280 g per plot) of bell pepper were produced. In the
290 second cropping season, *ca.* 50,000 seeds per plot (*ca.* 130 g per plot) of fresh market tomato and
291 *ca.* 74,000 seeds per plot (*ca.* 233 g per plot) of bell pepper were extracted. From the negative
292 control field plots, *ca.* 57,000 (*ca.* 149 g) of fresh market tomato seeds and *ca.* 39,000 (*ca.* 419 g) of
293 bell pepper seeds were extracted.
294 The WTG was 2.61 g, 2.60 g and 6.96 g for processing tomato, fresh market tomato and bell pepper
295 seeds, respectively.
296

297 **Seed analysis and contamination rate of seed lots**

298 Tomato and pepper seed lots produced during both cropping seasons were tested according to the
299 EPPO (2013) standard diagnostic protocol for *Xanthomonas* spp. Specifically, for each seed lot,
300 three seed samples (*n = 10,000*) were analysed by direct isolation and molecular assays, and the
301 results are presented in Table 2. For seeds produced during the first cropping season, Xv was not
302 detected by either PCR or direct isolation on GYCA and mTMB media from tomato seeds produced
303 in Italy and in Serbia. For pepper, Xe was consistently detected by PCR and recovered on mTMB
304 and GYCA media from all seed lots produced in the different field plots. The seed contamination
305 level was assessed as 34 (SD = 13), 37 (SD = 22) and 100 (SD = 48) CFU g^{-1} in the 1%, 5% and
306 15% infected plots, respectively. For seeds produced during the second cropping season, direct
307 isolation and molecular analyses did not result in the detection of Xv and Xe in any plot in Italy or
308 in Serbia.
309 Additionally, to determine the contamination rates of each seed lot, 50 replicates of 100 seeds were
310 analysed. The contamination rate of seed lots produced during the first cropping season in Italy and
311 Serbia was determined according to the formula $p = 1\text{-}(Y/N)^{1/n}$ (Maury *et al.* 1985). No seed

312 contamination (0%) was observed for processing or fresh market tomato produced in Xv-infected
313 plots. For pepper seeds, the seed contamination rate was 1.50%, 3.17% and 3.17% (Table 2) in the
314 plots infected with Xe at 1%, 5% and 15%, respectively. The seed contamination rate of all seed
315 lots produced during the second cropping season in Italy and Serbia tested negative for Xv and Xe.
316
317 **Seed germination rate assay**
318 All seed lots produced during both cropping seasons in Italy and Serbia were subjected to quality
319 testing by assessing their germination rate and their ability to produce marketable seedlings. The
320 germination rate of tomato and pepper seeds produced during the first cropping season was *ca.* 95-
321 98%, with no significant differences ($P \geq 0.05$) among seed lots produced in plots with different
322 disease quantities. Similar results were obtained from seed lots produced during the second
323 cropping year. No significant differences ($P \geq 0.05$) in the germination rate (ranging from 90 to
324 98%) were observed among the different seed lots produced and the uninfected plots (negative
325 controls).
326
327 **SGO assay**
328 The assessment of disease incidence during the SGO assays did not reveal any disease symptom on
329 seedlings obtained from seeds produced in both the first and second cropping years in Italy and
330 Serbia. Additionally, microbiological and molecular analyses performed on each seedling macerates
331 and their DNA extracts were all negative for the presence of Xv and Xe.
332
333 **DISCUSSION**
334 Xv and Xe are phytopathogenic bacteria of great concern to seed companies and farmers. Xv has
335 been recorded in several important seed-producing countries (*e.g.*, Brazil, Mexico, USA, India,
336 Thailand), whereas Xe exhibited a far more restricted distribution (*EFSA* Panel on Plant Health,
337 2014). Both Xv and Xe are seed-borne pathogens, and infested seeds serve as a main source of

338 primary inoculum in transplant and fruit production systems (Jones *et al.* 1993; Leite *et al.* 1995;
339 Dutta *et al.* 2014).
340 Seed companies are devoted to producing tomato and pepper seeds under pathogen exclusion
341 conditions, either in areas where the pathogens have never been reported, or under strict hygienic
342 practices (Gitaitis & Walcott, 2007). Nonetheless, disease symptoms may appear in seed production
343 fields due to the use of seeds that are contaminated at a level under the pathogen detection threshold
344 or because the production area is not sufficiently surveyed. It remains unknown whether a
345 consistent positive correlation exists between seed contamination rate and disease outbreak and
346 whether a similar correlation exists between disease quantity, as measured in fields devoted to seed
347 production, and the phytosanitary quality of the resulting seeds.
348 In our study devoted to simulate bacterial spot outbreaks occurring from external sources of
349 *inoculum*, the results obtained during the first cropping season revealed a positive correlation
350 between the incidence of initial infection, disease progression and disease quantity/score (Q) for
351 both Xv and Xe, as confirmed by the calculated AUDPC values. The disease scores measured on
352 tomato were remarkably different between the Italian and Serbian fields; these differences might be
353 explained by the length of the growing cycle, which is considerably longer for fresh market tomato
354 (7 weeks longer) than that for processing tomato, and by the presence of more favourable
355 environmental conditions for disease outbreak in Serbia than in Italy (*e.g.,* higher RH).
356 Similar disease progression occurred in bell pepper, since monitoring and harvesting of peppers
357 continued for an additional 8 weeks after processing tomato harvest. In addition, even though
358 AUDPC values from 1% infected plots appeared lower than those from 5% and 15% infected plots,
359 the differences were not significant. This could be due to the length of the growing cycle, but also to
360 the more effective spread ability of Xe in bell pepper in comparison to that of Xv in fresh market
361 tomato at the same environmental conditions.
362 In Italy, Xv infections produced spots on a limited number of fruits: this might be explained by the
363 low daily mean RH (< 60%), which was consistently measured at anthesis and fruit development

364 and ripening (July-August 2013). Conversely, during the same physiological growth stages in
365 Serbia, a daily mean RH > 80% was consistently recorded. Therefore, the RH conditions, which
366 were more favourable for bacterial infection than those recorded in Italy, might explain the high
367 disease incidence and severity expression on both fresh market tomato and bell pepper. Given that
368 no outside sources of *inoculum* were present during the field experiments, bacterial disease
369 development in the plots was the exclusive result of infection from the artificial *inoculum* sources,
370 as previously discussed by Kocks & Zadoks (1996) for other xanthomonads. Indeed, the appearance
371 of typical *foci* in the field plots around the experimental *inoculum* sources (experimentally
372 inoculated plants were marked) ultimately supports this conclusion (Zadoks & Van den Bosch,
373 1994). Finally, the pathogens were re-isolated from infected plants, and their identities were
374 confirmed by rep-PCR as the experimental *inoculum* sources (*i.e.,* strains IPV-BO 2684 and KFB29
375 of Xv and MI-A-6 of Xe).
376 Despite the positive correlation observed between the incidence of initial infection, disease
377 progression and AUDPC values for both Xv and Xe, the analyses of seeds produced during the first
378 cropping season did not exhibit any apparent correlation between disease quantity over time
379 (AUDPC), as measured in the production fields, and contamination rates of produced seeds for
380 either the tomato or pepper pathosystems. In the case of tomato, none of the seed analyses detected
381 Xv, though 58% and 63% of the total produced seeds in Italy and in Serbia were tested,
382 respectively, and the disease observed in the field plots was remarkably severe and present on all
383 aerial parts as leaves, fruits, petioles and stems. Conversely, seed contamination rates were between
384 1.50% and 3.17% for pepper seed lots produced in diseased plots. These pepper seed lots tested
385 positive for Xe detection by conventional PCR and by isolation on semi-selective media. These
386 results confirm a seed contamination by Xe; however, the infection is characterized by relatively
387 low rates and non-uniform distribution of Xe among the seed lots. Pathogen re-isolation indicated
388 that the seed population density for Xe was *ca.* 34 to 100 CFU g^{-1}.

389 The seed extraction protocol based on the fermentation of tomato pulp (Opeña *et al.* 2001) is
390 thought to play a major role in the viability and detectability of infecting bacteria. This method is
391 able to decrease the viability of bacterial populations contaminating the seeds, leading to a
392 consistent reduction in the density of viable bacterial cells on tomato seed surfaces (Chambers &
393 Merriman, 1975). Bacteria in the viable but non-culturable (VBNC) state, which fail to grow on the
394 routine bacteriological media, have been described (Oliver, 2005). These bacteria are in a state of
395 very low metabolic activity and do not divide. This feature occurs in response to stress (*e.g.,* due to
396 adverse nutrient, temperature, osmotic, oxygen, and light conditions) (Stokell & Steck, 2012). In
397 our case, we analysed seeds immediately after their production, and they were stored according to
398 the best possible procedures (*i.e.,* dry seeds stored at 4 °C in the dark). Therefore, we presume that
399 no stress or other adverse conditions may have caused the development of a VBNC status in both
400 Xv and Xe. An additional confirmation of this conclusion was obtained by molecular assays. These
401 assays could detect the DNA present in VBNC, but the results were negative for the presence of the
402 pathogen. Basically, when the pathogen was re-isolated on semi-selective medium, PCR
403 consistently detected its presence in DNA extracts.
404 Throughout the second cropping season, no bacterial spot symptoms were observed in pepper or
405 tomato plants. Moreover, in seeds extracted in the second cropping season, the pathogens were not
406 detected by any of three biologically different tests: microbiological, molecular and SGO assays.
407 Therefore, the lack of symptom development in all field plots indicates that seeds were not infected
408 or contaminated by Xv or that the pathogen transmission was negligible due to a low bacterial load
409 of Xe on pepper seeds, which was not sufficient to cause disease development in the next cropping
410 season. Since Bashan *et al.* (1982b) observed that Xv can survive in tomato seeds for a long time,
411 up to 8 years, we may presume that the viability of the pathogen in seeds used in the following year
412 was not dissimilar to the contamination level assessed after seed production.
413 In the past, *inoculum* transmission thresholds in seeds have been studied in few pathosystems
414 addressed to identify the correlation between seed infection rates and disease outbreaks, as in the

415 case of *Pseudomonas savastanoi* pv. *phaseolicola* in been seeds (Taylor *et al.* 1979) or
416 *Xanthomonas campestris* pv. *campestris* in crucifer seeds (Shaad *et al.* 1980). In these studies, the
417 *inoculum* thresholds were set either arbitrarily (*e.g.* using experimental seed inoculation) or simply
418 with field observations. In the case of tomato and pepper seeds, the correlation between *inoculum*
419 thresholds of Xv and Xe and disease outbreaks has not been previously evaluated under field
420 conditions. Additionally, to the best of our knowledge, no studies in the literature have elucidated
421 the quantitative/qualitative correlation between disease quantity observed in tomato/pepper fields,
422 the concentration of *inoculum* in the seeds produced in those fields and the bacterial spot outbreak
423 risks posed by these seeds on the next crop.
424 This is the first report on the evaluation of a seed contamination threshold for bacterial spot in
425 pepper. The results from this study showed that a seed contamination level higher than 100 CFU g^{-1}
426 is needed for a disease outbreak. Due to the highly polycyclic nature of the disease, it is important
427 to emphasise that the threshold level may be variable, considering that pepper growing areas have
428 quite different climatic conditions and/or different agronomic practices (*e.g.,* higher seeding rates).
429 Conversely, for the Xv-tomato pathosystem, our data showed a total lack of *inoculum* transmission,
430 confirmed by the absence of living cells on semi-selective media used for re-isolation. Xv, during
431 SGO assays and under field conditions, was not transmitted both to seedlings and to seeds in either
432 Italy or Serbia. The results obtained from molecular and microbiological assays on seed extracts of
433 processing tomatoes also suggest the important role of extraction protocols in seed sanitation (*i.e.*, a
434 fermentation step). The fermenting process could be considered an appropriate seed surface
435 disinfection step that does not affect seed quality, as confirmed by *in vitro* germination results. The
436 results obtained from SGO assays, in particular those related to the Xe-pepper pathosystem, for
437 which plant-to-seed transmission was successfully achieved, suggest that the bacterial loads in
438 contaminated seeds were not sufficient to develop symptoms, even using optimal controlled
439 conditions for both host and pathogen. As observed in the field, the pathogen population on seeds of
440 the two strains used were confirmed to be insufficient for disease outbreak.

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

444

445 **ACKNOWLEDGEMENTS**

446 This research was financed in the framework of the EU Project "Development of seed testing
447 methods for pests and pathogens of plant health concern (TESTA)" (www.seedtesta.eu). The
448 authors wish to thank Assosementi, Italian Member of the European Seed Association (ESA) and
449 the International Seed Federation (ISF) for assistance and for providing seeds for the experiments
450 and facilities for workshops and dissemination of results.

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545

Figures

Figure 1. Disease progression curves of bacterial leaf spot caused by *Xanthomonas vesicatoria* on processing tomato plants (cv. VF 10) from the beginning of July to the 1st of 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.

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.

567 **Tables**

568

569 Table 1. AUDPC values calculated for the different pathosystems in Italy and Serbia during the first
570 cropping season. Different letters indicate different statistical classes (Duncan's test, p 0.05).

571

572 Table 2. Contamination levels and rates of *Xanthomonas vesicatoria* (Xv) and *Xanthomonas*
573 *euvesicatoria* (Xe) in tomato and pepper seeds, respectively, produced in Italy and Serbia during the
574 first cropping season from field plots experimentally infected at different initial contamination
575 incidences (1%, 5% and 15%). The bacterial colonies (CFU) are related to 1.0 g^{-1} of seeds. To
576 determine the seed contamination rate of each seed lot, 50 replicates of 100 seeds were tested. Then,
577 their contamination rate (p) was calculated according to the formula $p = 1-(Y/N)^{1/n}$, where N is the
578 number of analysed replicates, n is the number of seeds in a replicate and Y is the number of non-
579 contaminated sub-samples (Maury *et al.* 1985).

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SUPPLEMENTARY MATERIAL

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 table 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 the 1st of April to the last harvest.

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