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SEED TRANSMISSION OF *XANTHOMONAS VESICATORIA* AND *CLAVIBACTER MICHIGANENSIS SUBSP. MICHIGANENSIS* IN TOMATO AND *XANTHOMONAS EUVESICATORIA* IN PEPPER AND IMPLEMENTATION OF SEED DISINFECTION METHODS

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ABSTRACT

Seed-borne bacterial pathogens of tomato and pepper are of major concern worldwide. *Xanthomonas vesicatoria* (Xv) and *Xanthomonas euvesicatoria* (Xe), the causal agents of bacterial leaf spot, and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), the causal agent of tomato bacterial canker, are worldwide distributed, but the occurrence of the latter is usually erratic. In order to evaluate the risk of seed transmission and the relationship between seed contamination and disease outbreak, an extensive field trial has been put in place in 2013 for each pathosystem. Three artificially contamination levels were considered (1%, 5% and 15% or 20%, respectively in Italy and in Serbia), composed of 100 seedlings each. Disease outbreaks were monitored weekly during the growing season until harvesting and disease was quantified by means of AUDPC. Seeds were produced from each plot and analysed in order to assess their contamination level. Preliminary results of our studies showed that disease quantity caused by Xv, Cmm or Xe was directly correlated to the percentage of initial infection, according to AUDPC values obtained. Contamination rate of seed produced in diseased fields was not always correlated with disease quantity observed. A microbial consortium, a bacterial antagonist and a plant polyphenols were assayed to assess their potential efficacy in seed disinfection: naturally contaminated tomato and pepper seeds were treated and sown. Pepper and tomato seedlings were inspected and analysed for the presence of bacterial spot. Preliminary results obtained show that none of the above mentioned treatments was able to eradicate the pathogen from seeds.

Key words: seed-borne bacteria, tomato, pepper, seed transmission, seed disinfection.

INTRODUCTION

Xanthomonas vesicatoria (Xv) and *X. euvesicatoria* (Xe) (Jones *et al*., 2004) are the causal agents of bacterial spot of both tomato and pepper. Long-distance dissemination of those xanthomonads is ensured by means of contaminated seeds in trade (Carmo *et al*., 2001). Bacterial spot is a widespread and economically very important disease of tomato and pepper. *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is the causal agent of tomato wilt and canker. The primary inoculum source for Cmm is contaminated seed (De

Leon *et al*., 2011), Chang *et al.* (1991) reported that one infected seed in 10.000 is able of initiating an epidemic. Cmm infections often result in high yield losses; in several cases, losses of between 50 and 100% have been reported. Xv, Xe and Cmm have been listed as A2 quarantine pests by EPPO.

This preliminary study was aimed to assess the impact of the diseases in the field, after an experimental inoculation with Xv, Cmm and Xe on tomato and bell pepper plants in different plots, and to evaluate the transmission rate from the infected plants to the seeds. In this study we also investigated the efficacy of different treatments, to reduce pathogen contamination in tomato and pepper seed, by treatments with a natural plant polyphenols, a microbial consortium and an antagonistic bacterium, specific for Xv.

MATERIAL AND METHODS

For each pathosystem, three fields of 96 plants each were set, and the plants of any field were randomly marked and inoculated in order to obtain 3 different percentages of initially inoculated plants: 1, 5 and 15% in Italy (industrial tomato), and 1, 5 and 20% in Serbia (table tomato and bell pepper). High susceptible cultivars to bacterial diseases were used: industrial tomato, cv. VF10; table tomato, cv. Jabučar and bell pepper, cv. Amphora. Experiments with Xv, Xe and Cmm were conducted in confined experimental field.

Experimental inoculation and phytopathometric evaluation

Tomato and pepper seedlings were transplanted in the fields, following the best good agricultural practices in place for Italy and Serbia. For tomato inoculation, strains IPV-BO 2684 of Xv (Italy), KFB29 of Xv (Serbia), DLS 598 of Cmm (Italy) and for pepper inoculation the strain MI-A-6 of Xe, were routinely grown on GYCA (Dye, 1962) for 48 hours at 27°C. Five weeks after transplanting, each plant was experimentally inoculated by spraying a water suspension containing the pathogen (ca. 10^8 CFU/mL). Each inoculated plant was sealed in a polythene bag (PE) overnight, which was removed the early next morning. The first phytopathometric readings were done at symptoms appearing and were carried out weekly. The disease severity of tomato and pepper plant affected by xanthomonads was evaluated using a descriptive scale ranging from 0 to 4: 0= no symptom; 1= 1-10 spots on 1-3 leaves; 2= 11-30 spots on 4-10 leaves; 3= more than 30 spots and some confluent necrosis on 5-20 leaves; 4= confluent necrosis on more than 20 leaves or branch desiccation. In case of Cmm infections, the disease severity on leaves (percentage of symptomatic leaves) was evaluated on each tomato plant on the basis of 5 disease severity classes (0, 5, 10, 25 and 50%). Disease score was calculated as $\sum$ of Q = Severity x Incidence. Area under the disease progress curve (AUDPC; Van der Plank, 1963) was then calculated according to Madden *et al.* (2007). Data were collected and statistically evaluated from the first observed symptoms to the last assessment before harvesting. In case of pepper and table tomato, readings were done during a longer time span, since harvesting was done gradually.

Crop harvest and seed extraction

Tomato and pepper seeds were produced according to common commercial procedures. For both tomato cultivars, the seed was extracted following the fermentation technique.

Seed analyses for the estimation of seed infection rate

For each pathosystem, ten samples of 100 seeds each, belonging to each infected field, were soaked in 3 mL of sterile PBS-Tween 20 (0,05%) for 14 hours at 4°C (see ISTA rules). The samples were then crushed for 2 minutes, the extraction liquid was centrifuged at 10.000 *g* for 20 min at 4°C and the pellet was resuspended in 2 mL of sterile PBS-Tween 20. DNA was extracted from seed macerates using the DNeasy Plant Mini kit (Qiagen) and assayed using the protocol of Koenraadt *et al.* (2009): Bs-XeF and Bs-XeR primer pair for Xe and Bs-XvF and Bs-XvR primer pair were used to detect Xv. DNA isolated from Cmm infected seed was assayed according to Dreier *et al.* (1995). The analyses were repeated 5 times in different days (5 replicates), for a total of 5000 seeds, in order to statistically assess the seed contamination rate.

Biological treatments of seed

Tomato seed, cv. Jabučar and pepper seed, cv. Amphora, naturally contaminated by Xv and Xe, respectively, were used. The following compounds were tested: a commercial microbial consortium and a commercial plant polyphenols on both tomato and pepper seeds, as well as a bacterial antagonist on tomato

seed. The microbial consortium (Micosat F, CCS Aosta, Italy) contained: *Glomus* spp., *Trichoderma* spp., *Agrobacterium radiobacter*, *Bacillus subtilis*, *Streptomyces* spp. Treatment was done according the manufacturer's indications: seed was dipped in a water suspension of the consortium, calculating 4.5 g/kg of seed. A commercial plant polyphenols based on tannins (AGRITAN, Silvateam, San Michele di Mondovì, Italy). Treatment has been done according the manufacturer's indications, by dipping seeds in a 10 g/L polyphenol solution in deionized water. A strain of *Pseudomonas synxantha* (DLS A65) active *in vitro* against Xv was preliminary assayed to control Xv on tomato seeds. Treatment has been done by dipping seed in a bacterial suspension of 10^8 CFU/ml. For treatments, seed was kept soaking for 90 min in a rotary shaker at 90 rpm, dried in an incubator at 30°C (with fan) overnight in the dark and stored in a seed storage room 1 month before sowing. Untreated seeds were used as a positive control.

Seed germination and disease assessment

In order to assess the effects of biological treatments on seed quality and its efficacy in seed sanitation, three replicates, consisting of 100 seeds for each treatment, were assayed in each of the three following tests. Germination *in vitro* was done according to ISTA rules. Seeds were placed on top of two layers Whatman n° 5 filter paper, moistured with 5 ml of sterile distilled water in Petri dishes. Petri dishes were placed at 25°C in the dark. Germination counts were assessed every day, up to 14 days. Germination test on blotter was carried out in a growing chamber, at 28-30°C and RH up to 75%. In pot tests, seeds were sown into pots containing a steam sterilized peat for seedling production. Growing chamber conditions were kept as above. Disease symptoms were daily monitored up to 28 days. In case of no symptoms development within 4 weeks, a stem segment (~2cm) of each seedling within the same replicate was collected and placed in a Stomacher Bag with 30 ml of sterile NaPBS buffer (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4, pH = 7.2). All the samples were crushed by hammering and stored at room temperature for 30 minutes. The washing fluids were then centrifuged and DNA was extracted by using DNeasy Plant Mini kit (Qiagen). The DNA was extracted from seed macerates using DNeasy Plant Mini kit (Qiagen) and assayed using the protocol of Koenraadt *et al.,* (2009). Primers used were: Bs-XeF and Bs-XeR for Xe and Bs-XvF and Bs-XvR for Xv.

Statistical analysis

All measurements were performed in triplicates. Analysis of variance (ANOVA, Tukey's test, $P \leq 0.05$) was applied using GraphPad Prism 6.0 software (La Jolla, California, USA).

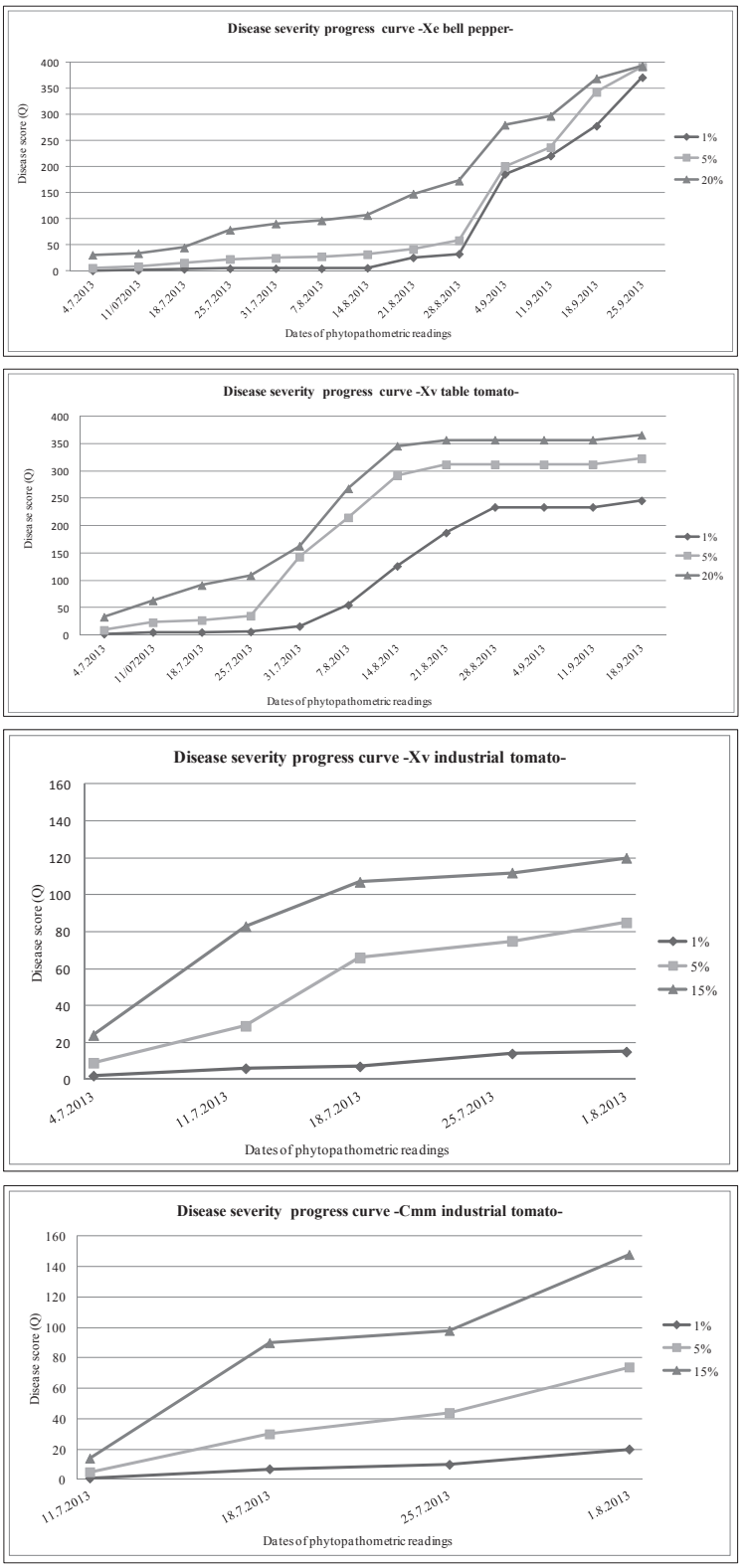
RESULTS

Phytopathometric evaluation of field experiments

For the experiments that were performed in Italy, the increase of the disease progression curve calculated for Xv and Cmm in industrial tomato was directly correlated to the percentage of initial infections; disease symptoms appeared 2 and 3 weeks after inoculation, respectively, and increased until the last survey (Graph 1). For industrial tomato plants inoculated with Xv, the AUDPC of the field with 1% initial infection was approximately six and ten times lower than that of the fields with 5% and 15% respectively. As regards the AUDPC obtained for Cmm from the field with 1% of initial infection, it was approximately four and ten times lower than that of the fields at 5% and 15%, respectively (Table 1). In Serbia, bacterial spot symptoms on table tomato and bell pepper appeared 2 weeks after the experimental inoculation and increased until the last survey (Graph 1). AUDPC value for Xv in the field at 1% of initial infections was approximately two times lower than that of fields at 5% and 20%. For Xe, the AUDPC referred to 1% of initial infection was approximately two and three times lower than that of the fields at 5% and 20%, respectively (Table 1).

Table 1. AUDPC values obtained in the different pathosystems considered (according to Madden *et al.* 2007).

Pathosystem	AUDPC values		
	Initial contamination rate (experimental)		
	1%	5%	20%
Xv-table tomato	8589	15074	18788
Xe-bell pepper	5743	8522	13632
	Initial contamination rate (experimental)		
	1%	5%	15%
Xv-industrial tomato	249	1512	2654
Cmm-industrial tomato	196	812	1932



Graphic 1. Disease severity progression curves over the time of each pathosystem considered in this study. In the legend, percentage value indicates initial percentage of inoculated plants per field.

Molecular analyses of seeds

The molecular analysis of seeds, by means of PCR protocol, did not result in the detection of Xv, in both industrial and table tomato (in Italy and in Serbia). On the contrary, seeds produced in Cmm contaminated plots were found to be all positive by PCR. Seed samples obtained from field plots inoculated with Xe at 1, 5 and 15% level resulted in PCR positive by 78, 96 and 96%, respectively.

Biological treatments of seed

Germination tests on blotter, performed with tomato and pepper seeds after biological treatments, did not show significant differences to the untreated ones. In *in vitro* experiment, the germination rate of tomato seeds treated with the microbial consortium and commercial plant polyphenols showed an apparent, but not significant increase compared to that of the untreated. The treatment with the bacterial antagonist DLS A65 significantly affected the germination rate of tomato seeds (Table 2). Germination tests *in vitro*, performed with pepper seeds after a treatment with the microbial consortium, was not different to untreated control, on the contrary, a treatment with commercial plant polyphenols decreased the germination by approximately 10%, if compared to untreated seeds. Such decrease was significant ($P \leq 0.05$). No symptom development was ever observed in both tomato and pepper seedlings until 28 days. Interestingly, PCR tests performed on same seedlings, confirmed the presence of Xv and Xe in tomato and pepper seeds, respectively.

DISCUSSION

In this study, we demonstrated during our field experiments a positive correlation between percentages of initial infection and disease progression and quantity caused by Xv, Xe and Cmm, as shown by the AUDPC value obtained. In addition, we highlighted differences in the AUDPC values obtained in industrial tomato fields and in table tomato plots: those differences might be explained by the length of cultivation, remarkably longer for table tomato (7-8 weeks longer) than for industrial tomatoes. The same for bell pepper, since monitoring and harvesting of peppers continued for additional 8 weeks, after industrial tomato harvesting day. Among the different pathosystems, contamination rates of tomato seed produced in affected plots were not correlated with disease quantity observed and measured in the fields. In particular, no contamination rate of Xv was found in both table and industrial tomato seeds, although the disease observed was remarkably severe and present on all aerial parts: leaves, fruits, petioles and stems. In contrast, pepper and tomato seeds, respectively produced in Xe and Cmm contaminated plots, were all found PCR positive. Further work is necessary to deeply investigate the pathogen transmission from plant to seed and from seed to plant by means the setup of extensive field trials using seed produced during this study. Further experiments are underway to assess the effect on the bacterial cells viability (Xv and Cmm) of the fermentation process during tomato seed extraction, which supposedly reduced the bacterial load.

Biological seed treatments with plant/fungal extracts apparently enhanced the germination rate *in vitro* and on blotter for tomato seed. On the contrary, no effect on the germinability was observed for pepper seeds. No bacterial spots occurred during the pot test on tomato and pepper seedlings; however, asymptomatic plantlets, collected and analyzed with PCR assays, showed that bacterial inoculum was present. Therefore, bacteria from seeds moved acropetally and colonised the seedlings: there they may survive as residents or increase the populations in seedlings until they reach the leaves without causing symptoms (Silva *et al*., 2013). Results of the biological seed treatments showed that they were not effective

Table 2. Germination rate *in vitro* and on blotter of tomato seed cv. Jabučar and pepper seed cv. Amphora after treatments. Different letters within columns denote significant differences according to the Tukey's test ($P \leq 0.05$).

	Treatment	Germination (%)	
		in vitro	blotter
Tomato cv. Jabučar	Microbial consortium (Micosat F, CCS Aosta, Italy)	98.67^{A}	96.67^{A}
	Plant polyphenols (AGRITAN, Silvateam, Italy)	92.67^{A}	91.67^{A}
	Bacterial antagonist	78.33^{B}	$80,67^{A}$
	Untreated	86.67^{AB}	85.67^{A}
Pepper cv. Amphora	Microbial consortium (Micosat F, CCS Aosta, Italy)	97.00^{A}	92.00^{A}
	Plant polyphenols (AGRITAN, Silvateam, Italy)	84.00^{B}	85.67^{A}
	Untreated	96.33^{A}	85.67^{A}

in eradicating the pathogenic bacteria associated with seeds. Further studies are needed to check, if such plant polyphenols or beneficial microbes might have a role in inducing of resistance. They might also enhance the germinability and the performance of seeds. Additionally, they could be taken into consideration to increase plant productivity of tomato and pepper crops. Nevertheless, new approaches in sanitation methods are needed to ensure efficient seed sanitation/disinfection, together with an optimization of formulations and application procedures related to such innovative bioproducts.

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