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IDENTIFICATION OF *XANTHOMONAS* SPP. DISEASE AGENT/S AND THE EFFECT OF CHEMICAL SEED TREATMENTS TO CONTROL BACTERIAL SPOT OF PEPPER

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ABSTRACT

Bacterial leaf spot caused by the xanthomonads is a destructive disease of both tomato and peppers. This paper aimed to identify the causative bacterial spot agent/s of pepper in Kayseri Province of Turkey. Plants of pepper with leaf lesions picked up among pepper plants in fields. Xanthomonad-like yellow pigmented 12 bacterial strains obtained from infected leaf samples. These strains were identified using phenotypic features like gram reaction, oxidative metabolism, growth at 40°C and on YDC medium, amylolytic and pectolytic activities. The molecular identification completed with species specific primers. All strains were gram negative, oxidative metabolism and grew at 40°C, produced yellow mucoid colonies on YDC medium and showed strong amylolytic activity, while all did not have any pectolytic activity. All strains characterized as *Xanthomonas euvesicatoria* according to phenotypic and molecular characterization. The study concluded that *X. euvesicatoria* is the prevalent causal agent of pepper bacterial spot in Kayseri province. The second aim of the study was to evaluate the potential use of chemical seed treatments including sodium hypochlorite, cupric acetate, streptomycine sulphate, acetic, citric and lactic acid in the elimination of pepper bacterial spot disease. Two experimental groups were thus designed: 1) efficacy of treatments on the survival of *X. euvesicatoria* on pepper seeds was observed 2) the effects of seed treatments on germination and disease development on seedlings at chamber room conditions were evaluated. The treatments were used in both experiments were negative (with water only) and positive (only pathogen) control, immersion seeds into sodium hypochlorite (3%, 1, 3 or 5 min), streptomycine sulphate (0,02 g per liter), cupric acetate (0,2% 1, 3 or 5 min), acetic and citric acid (1% 1, 3 or 5 min) and lactic acid (2% 1, 3 or 5 min). All treatments were statistically significant. The efficacies of treatments on disease incidence and severity were greater than 89%. Additionally, these treatments had no adverse effect on seed germinations. The study recommended seed treatments with sodium hypochlorite (3%, 3 and 5 min), copper acetate (0.2%, 3 and 5 min), acetic and citric acid (1%, 1, 3 and 5 min) and lactic acid (2%, 1, 3 and 5 min) as the most suppressive treatments to control bacterial spot disease symptoms on pepper. The seed treatments have potential to control the disease in nurseries.

KEYWORDS:
Pepper, control, seed, occurrence, plant disease

INTRODUCTION

Pepper (*Capsicum annuum*) is grown on over 47 466 ha in Turkey with a total annual production of 1 528 million tons [1]. In Turkey, pepper is consumed as fresh vegetable and prosessed pepper paste and powder for cooking or spicing foods. Bacterial spot, caused by *Xanthomonas* spp., is distributed one of the main diseases of pepper around the world [2]. It occurs predominantly during the rainy season with high temperatures [3]. The presence of the disease has been reported from all continents of the world including Africa [4], America [5], Asia [6], Australia [7] and Europe [8]. In Turkey, disease has been reported in tomato [9, 10] and pepper [11] producing regions. Based on DNA-DNA hybridization, four genetically distinct species, namely *Xanthomonas euvesicatoria, X. vesicatoria, X. perforans* and *X. gardneri* are reported as the causal agent of bacterial spot of pepper and tomato [5]. In Turkey, *Xanthomonas euvesicatoria* and *X. perforans* have been reported on both pepper and tomato [12].

Plant debris, infected seeds and volunteer plants may serve as inoculum sources of the disease [13]. The bacterial spot pathogens can infect all aboveground parts including pepper leaves, petals, stems and fruit stalk. The spots are water-soaked during rainy periods. Yield reductions can result from defoliation, leaf abortion and fruit damage [14]. In several countries including Turkey, copper-mancozeb combinations are widely used in disease control. Chemical control of the disease is a challenge due to development of copper and streptomy-

cin resistant bacterial spot pathogen populations ([15, 16], high variability of the causative agent/s and presence of races [5]. Since the pathogen is seed-borne, survives in infected plant debris, presence of copper-resistant *Xanthomonas* spp., populations, bacterial spot management is collapsed. Cultural practices do not provide a sufficient reduction of the disease. Thus, a range of disease management strategies must be integrated [17].

Three single dominant genes designated as *Bs1, Bs2* and *Bs3* have been identified for resistance to the pathogen [18]. None of these genes confers resistance to all *Xanthomonas* strains in pepper. In Turkey, plant breeding based attempts are increased, however, most of newly developed pepper varieties in the market are susceptible to bacterial spot. Rotation of fields may help to avoid transmission of inoculum on volunteer plants and crop residue. Disease is getting common in nurseries both on pepper and tomato as a result of high humidity, a large amount of seedlings have to be destroyed due to bacterial spot infection. The critical control measurement in nurseries is to use pathogen-free seeds or reduce pathogen inoculum on/in seeds.

For decades, seed treatments are highly recommended and employed to eradicate or reduce pathogen transmission [19]. Natural antimicrobial compounds can be used for seed disinfection as an alternative treatment. Essential oils and/or extracts of some plants like *Allium sativum, Eucalyptus* sp. *Rosmarinus officinalis, Satureja hortensis, S. spicigera, Origanum onites* and *O. rotundifolium* have a potential to control seed infestation of *Xanthomonas* spp. in pepper and tomato [20, 21]. The organic acids, acetic acid and lactic acid were effective for control of wheat common bunt in reducing infection levels [22, 23]. Bacteriophages, biological control agents and SAR inducers have been successfully used for managing tomato plant bacterial diseases [24, 25, 26, 27, 28].

In the first aim of this study, researcher has identified the causal agent/s of pepper bacterial spot in Kayseri Province of Turkey using phenotypic and molecular test procedures.

The use of healthy seeds is the most important factor for controlling the seed-borne diseases. Therefore, this paper has assessed the efficacy of chemical seed treatments against *Xanthomonas* spp. both on pathogen development and seed germinations.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The 12 strains of *Xanthomonas* spp. were obtained from pepper producing fields in Kayseri Province, Turkey during the years of 2016 and 2017. Strains were grown on King's Medium B (KB) and incubated at 28 °C for 48 h. For long periods, strains were kept in 30% glycerol (v/v) at -80 °C.

Phenotypic and molecular characterization of *Xanthomonas* spp. strains. The putative xanthomonad strains were subjected to standard biochemical and physiological tests [3, 5, 29]. All strains were tested for gram reaction, oxidative metabolism, growth at 40°C and on YDC medium, pectolytic and amylolytic activities. All the biochemical tests were repeated once.

DNAs of bacterial strains were extracted from a freshly grown bacterial culture on NA medium for 48 h at 28 °C. DNA was isolated with the aid of DNeasy Blood &Tissue Kit (Qiagen, GmbH Germany) according to manufacturer's instructions. Molecular characterization was determined by PCR with species-specific primer pairs Bs-XeF/Bs-XeR, Bs-XvF/Bs-XvR, Bs-XgF/Bs-XgR and Bs-XpF/Bs-XpR for *X. euvesicatoria*, *X. vesicatoria, X. gardneri* and *X. perforans*, respectively [30]. PCR reactions were performed in a final volume of 25 µl containing 12,5 µl of DreamTaq master mix (Thermofisher Scientific, Vilnius, Lithuania), 1 µl of each primer (10 pmol/ µl), 1 µl genomic DNA and 9,5 µl Milli-Q water and the PCR amplification programmes were based on [31].

Pathogenicity tests. Pathogenicity tests were conducted on local pepper cultivar Bursa Yaglik 016 and tomato cultivar H-2274 plants. For each strain, three tomato and pepper plants were inoculated. The bacterial suspension ($1x10^6$ CFU/ml) was prepared from 48-h-old culture grown on YDC medium. Suspension was sprayed to the pepper and tomato leaves, petioles and stems until runoff. Sterile distilled water was used as negative control. All inoculated plants were subsequently incubated at 27±3°C and with 85–95% relative humidity in a chamber room to favour symptom development. The pathogenicity tests were repeated once.

Pathogen, growth conditions and inoculum preparation. *Xanthomonas euvesicatoria* strain SH-5 was used in this study for seed inoculations. The strain was grown on KB and incubated at 25 °C for 48 h. *X. euvesicatoria* suspension was generated in saline buffer (NaCI 0.85%) and the concentration was adjusted to A_{600}:0.2 OD with a spectrophotometer (Shimadzu UV-120-01, North America). The bacterial suspension was ten-fold serially diluted with saline buffer and 100 µl aliquots were spread onto KB. The concentration of the bacterial inoculum was estimated to be $3.6x10^8$ CFU ml^{-1} and this concentration was used in further studies.

Inoculation of pepper seeds and chemical seed treatments. Pepper seeds of the susceptible cultivar BT Bursa Yaglik 016 (Bursa Seed, Bursa, Turkey) were immersed into 250 mL of a *X. euvesicatoria* strain SH-5 suspension for 30 min and fil-

tered through double-layered cheesecloth. Seeds were air dried at room temperature (25±2°C) for 24 h in a laminar flow and divided into three lots (100 seeds/lot) before seed treatments. The chemical treatments applied to pepper seeds and the exposure times were as follows (per 0.1 liter sterilized water): streptomycin sulphate, 0.02 g for 5 min; cupric acetate, 0.2% for 1 3 or 5 min; sodium hypochloride, 3% for 1, 3 or 5 min; acetic acid 1% for 1, 3, or 5 min; citric acid 1% for 1, 3, or 5 min and lactic acid 2% for 1, 3, or 5 min. The solutions were freshly prepared before immersion of pepper seeds. Each sample (n=300 seeds) was rinsed as previously described and treated seeds transferred to dry in a laminar flow hood for 24 h at room temperature.

Efficacy of treatments on the survival of *X. euvesicatoria* on pepper seeds. The colonization ability of *X. euvesicatoria* was compared on treated and untreated seeds. Initially, *X. euvesicatoria* strain SH-5 was gradually maintained resistant to rifampicin antibiotic (100 mg/l). Pepper seeds (n=17 g) were inoculated with pathogen suspension as previously described. Two days after inoculation, each sample (n=1 g) of pathogen-inoculated seeds was treated with the chemicals as described above. Negative control pepper seeds (n=1 g) were treated with sterile distilled water. All treated pepper seeds were shaken on a shaker at 200 rpm for 30 min and air-dried in a laminar flow cabinet for 24 h. Each seed sample was put into glass tubes containing 9 ml sterilized nutrient broth and shaken at 250 rpm for 3 h. Each treatment was ten-fold serially diluted with nutrient broth including 1/10, 1/100, 1/1000 and 1/10 000 serial dilutions. Aliquots of 100 µl were spread onto KB medium supplemented with rifampicin, 100 mg/l, and incubated at 25°C for 4-6 days. Bacterial colonies were counted and the results were compared with positive control. Mean bacterial populations were calculated using the plate-count technique (number of colonies*dilution of sample*10).

Efficacy of seed treatments at chamber room conditions. Artificially inoculated and treated pepper seeds (n=150 seeds per treatment) were sown in small plastic pots 20x12x3 cm (lengthxwidthxheight) containing sterilized soil and incubated under climatic room with the following conditions: 25-28°C, 75% humidity and 16 h of light alternating with 8 h of darkness. Twenty days after planting, each cotyledon and true leaves were examined for spot symptoms. Bacterial spot seedling transmission (number of seedlings displaying typical bacterial spot symptoms divided by the number of seedlings that germinated*100) was recorded. Disease severity was evaluated visually and scored using a modified range of 0 to 3 (0 shows a healthy-looking plant; 1 signifies 1-2 spot on cotyledon; 2 signifies 3-4 spots; and 3 signifies more than 5 spots), as previously described [32]. Efficacy of seed treatments on seed germination percentage (number of germinated seedlings divided by the number of seeds that sown*100) were also determined. After germination, re-isolations completed from disease-infected seedlings to reveal the causative agent as *X. euvesicatoria*.

Experimental design and statistical analysis. The seeds subjected to chemical seed treatments were randomly placed in a growth chamber. Each treatment was replicated three times and each plastic pot containing fifty seeds was used per replicate. Data from each pot was combined and the entire experiment was analyzed by analysis of variance (ANOVA) at $P \leq 0.05$. The efficacy of treatments was evaluated using the Abbott formula (control-treated/control*100). All experiments were repeated twice in controlled conditions.

RESULTS

Phenotypic and molecular characterization of *Xanthomonas* spp. The 12 strains of *Xanthomonas* spp. from pepper were gram negative, had oxidative metabolism and grew at 40°C. All of them produced yellow mucoid colonies on YDC medium and showed strong amylolytic activity, while the strains did not have any pectolytic activity. PCR results showed the amplification of a DNA fragment of 173 bp with primers BS-XeF/BSXeR, specific for *X. euvesicatoria*, for all characterized strains. There was no amplification for the primers BS-XvF/BS-XvR, BS-XpF/BS-XpR and BS-XgF/BSXgR specific for *X. vesicatoria, X. perforans* and *X. gardneri*, respectively. Based on these results, it was concluded that *X. euvesicatoria* is the prevalent causal agent of pepper bacterial spot in Kayseri province.

Pathogenicity tests. The plants were periodically monitored for symptom development up to 4 weeks. Typical bacterial spots developed on both tomato and pepper plants three weeks after inoculations. Koch's postulates were accomplished by performing re-isolations of the inoculated strains on KB medium, and their identity was checked using species-specific polymerase chain reaction (PCR) primers. No symptoms appeared on control plants.

Efficacy of treatments on the survival of *X. euvesicatoria* on pepper seeds. The forming colonies on plates were recorded after five days of the seed treatments. In control plates, 102 mean bacterial colonies were counted on three ten-fold diluted petri dishes. Seed treatments conducted with sodium hypochlorite (3%, 1 min), copper acetate (0.2%, 1 min), and streptomycine sulphate reduced bacterial colonization on seed compared to control plates

(Table 1). No bacterial growth was observed on the plate dilutions of sodium hypochlorite (3%, 3 and 5 min), copper acetate (0.2%, 3 and 5 min), acetic acid, citric acid and lactic acid treated pepper seeds.

Efficacy of seed treatments at chamber room conditions. All chemical seed treatments significantly reduced bacterial spot development on pepper seedlings (Table 1). Seed treatments; sodium hypochlorite (3%, 3 and 5 min), copper acetate (0.2%, 3 and 5 min), acetic acid (1%, 1, 3 and 5 min) citric acid (1%, 1, 3 and 5 min) and lactic acid (2%, 1, 3 and 5 min) were totally suppressed bacterial spot growth in both experiments. Disease incidence and severity were 62.50% and 56.10% in positive control seedlings. Foliar disease incidence in treated seeds ranged from 0.00% to 6.48% and severity ranged from 0.00% to 4.08%, respectively. The efficacies of treatments on disease incidence and severity were greater than 89%. All treated seeds displayed 91.33% to 100% germination and treatments did not significantly reduce seed germinations (Table 1). According to both studies, seed treatments with sodium hypochlorite (3%, 3 and 5 min), copper acetate (0.2%, 3 and 5 min), acetic acid (1%, 1, 3 and 5 min), citric acid (1%, 1, 3 and 5 min) and lactic acid (2%, 1, 3 and 5 min) were the most suppressive treatment reaching to 100% both in bacterial colonization and seed treatments (150 seeds per treatment).

DISCUSSION

This study reports the occurrence bacterial spot of pepper in Kayseri province and characterization of the causal agent, *X. euvesicatoria* for the first time. Like our study [12] characterized this pathogen in her Msc thesis as the prevalent agent among eight out of 10 strains tested. Some phenotypic characteristics of the pepper strains were not similar to the overall description of the species. The amylolytic and pectolytic activity features were different. All tested strains have shown amylolytic activity, but no pectolytic activity. The type strain of *X. euvesicatoria* isolated from pepper in the USA [5] was weakly amylolytic and pectinolytic, while most of the pepper strains from Grenada [33] and Brazil [34] did not have any amylolytic and pectinolytic activity. However, [35] and [3] reported that some *X. euvesicatoria* strains could hydrolyse starch similar to the results obtained in our study. The absence of pectolytic activity of *X. euvesicatoria* strains was previously reported also from Brazil [34], Iran [6] and USA [5]. Contrary to this study, few *X. euvesicatoria* strains isolated from pepper in Grenada showed pectolytic activity [33]. All those results conclude that *X. euvesicatoria* strains are characterized by a high phenotypic diversity, thus, these features are not helpful to fulfill the identification of the strains.

Results of pathogenicity tests showed that pepper strains can infect tomato plants as described previously [5]. This has a great importance for epidemiological aspects since tomato and pepper are widely produced in Turkey and in the same area for years. Also *X. euvesicatoria* can survive on other crops like common bean and nightshade plants which can serve as an important alternative hosts for the pathogen [6]. For this reason, common bean is not suitable for a rotation and cultural practices are so important in/around production areas.

TABLE 1
Colonization of bacterial spot on each one g of pepper seeds and efficacy of seed treatments evaluated by disease incidence and severity (Means of two experiments)

Treatments	Cells/g seed	Incidence%	Efficacy%	Severity%	Efficacy%	Germination%
Negative control						100a
Positive Control	$1.04*10^6$	56.10a		62.51a		94.67a
Streptomycin sulphate	$2*10^3$	0.48b	99.15	6.48b	89.63	92.00a
Cupric acetate, 0.2% 1 min	$3{,}9*10^5$	0.97b	98.26	6.02c	90.37	91.33a
Cupric acetate, 0.2% 3 min	0.00	0.00c	100	0.00d	100	98.67a
Cupric acetate, 0.2% 5 min	0.00	0.00c	100	0.00d	100	94.67a
Sodium hypochloride 3% 1 min	$1*10^3$	2.81b	94.99	6.17b	90.12	94.00a
Sodium hypochloride 3% 3 min	0.00	0.00c	100	0.00d	100	97.33a
Sodium hypochloride 3% 5 min	0.00	0.00c	100	0.00d	100	100.00a
Acetic acid 1% 1 min	0.00	0.00c	100	0.00d	100	96.00a
Acetic acid 1% 3 min	0.00	0.00c	100	0.00d	100	92.00a
Acetic acid 1% 5 min	0.00	0.00c	100	0.00d	100	94.00a
Citric acid 1% 1 min	0.00	0.00c	100	0.00d	100	98.00a
Citric acid 1% 3 min	0.00	0.00c	100	0.00d	100	96.67a
Citric acid 1% 5 min	0.00	0.00c	100	0.00d	100	95.33a
Lactic acid 2% 1 min	0.00	0.00c	100	0.00d	100	95.33a
Lactic acid 2% 3 min	0.00	0.00c	100	0.00d	100	96.00a
Lactic acid 2% 5 min	0.00	0.00c	100	0.00d	100	98.00a

* Means followed by the same letters within each column are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test

PCR results showed the amplification of 173 bp DNA fragment with *X. euvesicatoria* specific primers. Most of the researchers have the similar results indicating that the pathogen is predominant [34, 6]. Since the first report of pepper bacterial spot in Turkey [11], there is no comprehensive study about all *Xanthomonas* species. No other tomato and pepper infecting *Xanthomonas* species observed in this study. It can be explained that the strains were isolated from a restricted area, thus, future studies with strains from other cities of Turkey and worldwide will help for an extensive genetic diversity.

Bacterial diseases of tomato are the major economic threat to this crop worldwide. Bacterial spot is seedborne and seriously affect both tomato and pepper crop yield and quality. Since the seeds are the primary inoculum sources, seed treatments have gained importance to control the disease. In this study, all tested chemical seed treatments significantly reduced bacterial spot development on pepper seedlings. Citric acid is used to control yeasts and bacteria in human health [36, 37], plant pathogenic fungi [38] and some tomato infecting pathogenic bacteria like *Clavibacter michiganensis subsp. michiganensis* and *Pseudomonas syringae* pv. *tomato* [39]. Recently, [40] evaluated the bactericidal activity of a commercial compound, Dentamet, containing zinc and copper complexed with citric-acid hydracids against the bacterium *Xylella fastidiosa* subsp. *pauca* in Italy. The authors suggest to include this product as spring and summer spray treatments to the integrated management to reduce severity of the pathogen. No published paper is in hand about the inhibition potential of citric acid on bacterial spot of pepper. The present study have revealed that citric acid at the ratio of 1% have a potential to control bacterial spot as seed treatment in nurseries and non phytotoxic. Citric acid should test *in vivo* for further information on its efficacy. It is cheap, easy to treat seeds and non-irritated to appliers. These promising results led scientists and farmers to include citric acid to control other plant threating microorganisms.

Cupric acetate acidified in acetic acid (0.25% or 0.5%) eradicated *Cmm* from the tomato seeds and soaking seed at 52 °C for 20 minutes with acetic acid (0.25% or 0.5%) resulted in the best reduction of saprophytic bacteria on/in tomato seeds. The scientists recommended this treatment (0.5%) as seed treatment because commercial seedlots may vary in their response to the treatments, that's why a small sample should be tested in order to check the adverse effect on the germination [41]. Since acetic acid treatment (1%) did not have any adverse effect to pepper germination and eliminated pathogen from pepper seeds, efficacy of cupric acetate (%0.2) has varied. This can be explained with the low dosage of cupric acetate, bacterial resistance to copper compounds [16].

Organic acids like acetic acid, citric acid and lactic acid were effective in reducing infection levels caused by plant pathogenic fungi and bacteria [22, 42, 43]. Like our study, [44] found no significant effect on bacterial count on seeds with the tested organic acids and the reduction was more than 99%. Moreover, seed germination qualities were not significant [45] tested apple and raisin vinegars (acetic acid) as organic acids to control bacterial wilt infected tomato seeds. The authors recommended these seed treatments to be used in organic and conventional farming systems. Our study advised all seed treatments except streptomycin to be used in both growing systems to eliminate bacterial spot from pepper seeds.

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