

Copper Resistance and Race Distribution of *Xanthomonas campestris* pv. *vesicatoria* on Pepper in Korea

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한국에서의 고추 더듬이병균의 구리 저항성과 레이스 분포

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ABSTRACT : A total of 66 strains of *Xanthomonas campestris* pv. *vesicatoria* were isolated from pepper leaves all over Korea in 1993 and 1994. Twenty-four strains of them were identified as race 1 and the remaining 42 as race 3, but race 2 was not found. Of all the strains tested, 8% were resistant to copper sulfate (200 µg/ml; 1.25 mM) and only one strain was resistant to streptomycin sulfate (100 µg/ml). Twenty-one % of race 1 strains were resistant to copper, but all strains of race 3 were sensitive to copper. One µg/ml (6.26×10^{-6} M) of copper sulfate in solution killed all cells of the sensitive strains or reduced viability to very low levels in 4 hr of exposure, but the viability of resistant strains appeared undiminished even in 128 µg/ml when compared with the control.

Key words : bacterial spot of pepper, race, copper resistance, streptomycin resistance.

Xanthomonas campestris pv. *vesicatoria* (Doidge) Dye is the causal agent of bacterial spot of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.). This disease can cause significant losses of yield, particularly in warm and humid environments (10, 17), and is greatly increased in severity by driving rains and by wounds due to hail or blowing sand (27).

Three groups of the bacterium, namely the pepper group (XcvP), the tomato group (XcvT), and the pepper-tomato group (XcvPT), have been described (15). XcvT is virulent on tomato and avirulent on pepper, whereas XcvP is virulent on pepper but not on tomato. Pepper and tomato are susceptible to XcvPT. Strains belonging to XcvP and XcvPT are further divided into a number of races on the basis of virulence on differential near-isogenic pepper lines (15). Resistance to bacterial spot of pepper is conferred by single dominant genes, *Bs1*, *Bs2*, and *Bs3* (11). Genes *Bs3* and *Bs1* offer resistance to race 1 and 2, respectively, and the *Bs2* gene restricts

races 1~3. Races 1 and 2 of XcvT also have been differentiated on tomato cultivars Walter and Hawaii 7998 (20).

The relatively low cost and low toxicity to mammals of fixed copper compounds give them an advantage over other chemicals for control of foliar bacterial diseases. Sprays of fixed copper have been recommended for control of bacterial spot of pepper since the disease was first described in 1922 (12). Copper is commonly used to control this disease. However, strains of this bacterium resistant to copper-containing compounds have been reported in Florida (14), and other locations (1, 3, 4, 6, 18, 28). Also, copper resistance occurs in several pathovars of *Pseudomonas syringae* van Hall (2, 7, 24) and some non-pathogenic bacteria (8). Streptomycin resistance in *X. campestris* pv. *vesicatoria* was observed in the late 1950s (23, 26).

An association between copper resistance and race 2 of the pepper strain of *X. campestris* pv. *vesicatoria* was noted (14). Loci for copper resistance and avirulence to pepper plants that have the *Bs1* gene for resis-

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tance to bacterial spot are linked and both loci were transferred with a large self-transmissible plasmid (22). Copper resistance in *X. campestris* pv. *vesicatoria* XV 81-23 is associated with the large (approximately 200 kb) self-transmissible plasmid, pXvCu (22). Other large plasmids in *X. campestris* pv. *vesicatoria*, including pXV10A, share homology with the pXvCu-type plasmids, but pXV10A does not show homology to IS476, which is present on the pXvCu-type plasmids (3).

The purpose of this research was to determine the presence of copper and streptomycin-resistant strains and race distribution of *X. campestris* pv. *vesicatoria* on pepper in Korea.

MATERIALS AND METHODS

Bacterial strains. In 1993 and 1994, *X. campestris* pv. *vesicatoria* was isolated from infected pepper plants received from several countries or collected from diseased plants during field trips.

Leaves of pepper with typical symptoms were surface-sterilized by dipping in 75% ethanol for 20 sec, followed by a 30–60 sec rinse in 1% sodium hypochlorite. Lesions were excised and crushed in 1 ml of sterile distilled water, and several loopfuls of the extraction mixture were streaked on YDC medium (10 g of yeast extract, 10 g of dextrose, 5 g of calcium carbonate, and 15 g of agar in 1 liter of distilled water) and incubated at 28°C. After 36–72 hr, colonies of *X. campestris* pv. *vesicatoria* were selected on a basis of morphology, color, and relative frequency of the colony type. Three to five colonies were pooled and streaked on YDC medium. Cultures were stored in sterile distilled water at room temperature until used for experiments.

Several standard determinative tests for *Xanthomonas* were used to characterize the bacteria, including cell morphology, Gram stain reaction, catalase, oxidase, nitrate reductase, acid production from sugars, and utilization of asparagine as a sole source of carbon and nitrogen (19).

To determine the pathogenicity of the bacterial strains, pepper plants (cv. Early Cal Wonder (ECW)) at the sixth to seventh true-leaf stages were used. To prepare inoculum, bacteria were grown on Luria-Bertani (LB) broth (10 g of bactotryptone, 5 g of yeast extract, and 10 g of sodium chloride in 1 liter of distilled water) for 12 hr at 28°C, and spectrophotometrically ad-

justed to 1×10^8 colony-forming units (cfu)/ml (optical density of 0.3 at 600 nm). The abaxial surfaces of the fourth and fifth true leaves of two pepper plants per strain were infiltrated with the bacterial suspension using a syringe with no needle (25). After inoculation, plants were kept in a greenhouse at 22–24°C under natural light conditions. Pepper plants infiltrated with sterile water served as controls. The strain Xcv 71-21 (pepper race 1) of *X. campestris* pv. *vesicatoria* was used as a reference strain for biochemical, nutritional, and pathogenicity tests.

Race determination. Races were determined using the procedures described by Minsavage *et al.* (15, Table 1). Bacteria were grown on YDC medium for 48 hr, suspended in sterile distilled water, and adjusted to about 1×10^8 cfu/ml. Leaves of the differential pepper cultivars ECW, ECW-10R, ECW-20R, and ECW-30R were infiltrated with bacterial suspensions using a syringe with no needle. Plants were incubated in the greenhouse at 22–24°C under diurnal light. Symptom development was observed until the seventh day after inoculation. In incompatible interactions, characterized by hypersensitive reaction, cell collapses and necrosis appeared 12–36 hr after inoculation; while in compatible interactions, water-soaked and chlorotic lesions were evident 3–5 days after inoculation. The pepper strain Xcv 71-21 (race 1), strain Xcv E3 (race 2) of *X. campestris* pv. *vesicatoria* were used as reference cultures.

Sensitivity to copper and streptomycin. Sensitivity of bacterial isolates to copper and streptomycin was assayed as described by Ritchie and Dittapongpitch (18). Sensitivity was tested on SPA medium (20 g of

Table 1. Races of *Xanthomonas campestris* pv. *vesicatoria* classified according to reactions of pepper differential cultivars^a

Pathotype	Reaction ^b of differential cultivars			
	ECW ^c	ECW-10R ^d (Bs1)	ECW-20R (Bs2)	ECW-30R (Bs3)
Race 1	S	S	HR	HR
Race 2	S	HR	HR	S
Race 3	S	S	HR	S

^a From Minsavage *et al.* (15).

^b HR : hypersensitive reaction; S : susceptible.

^c ECW refers to pepper cv. Early Cal Wonder.

^d ECW-10R refers to a line derived from ECW containing the resistance gene, *Bs1*.

sucrose, 5 g of peptone, 0.5 g of dibasic potassium phosphate, 0.25 g of magnesium sulfate, and 15 g of agar in 1 liter of distilled water) amended with appropriate chemicals. Fresh stock solutions of copper (copper sulfate, Shinyo Pure Chemicals Co., LTD.) and streptomycin (streptomycin sulfate, Sigma, St. Louis, MO) were prepared in sterile distilled water, filter-sterilized (0.45 μm pore size), and appropriate concentrations were added to SPA medium after autoclaving and cooling to 45°C before pouring into Petri plates. In media containing copper, acidity was adjusted with 2 N NaOH to pH 7 before autoclaving and again checked before sensitivity assays were done.

Bacterial cultures were grown on YDC medium or SPA medium for 36–72 hr and suspended in sterile distilled water, and the concentration was adjusted to 10^8 cfu per milliliter. Five microliters of bacterial suspension for each strain was spotted on SPA medium and SPA medium amended with either 200 $\mu\text{g}/\text{ml}$ (1.25 mM) of copper sulfate or 100 $\mu\text{g}/\text{ml}$ of streptomycin sulfate. Plates were incubated at 28°C for 48 hr, and the presence or absence of growth was recorded. Bacterial strains that grew on SPA medium amended with either copper sulfate or streptomycin sulfate were considered resistant to copper or streptomycin, respectively.

The relative resistance of strains to copper were confirmed by an additional quantitative test. Incubated bacterial strains on YDC medium for 48 hr were resuspended in sterile buffered saline to approximately 2.0×10^8 cfu/ml. Five ml of each bacterial resuspension was mixed in culture tubes with 5 ml of a 2.0 g/l suspension of a commercial copper hydroxide bactericide (Kocide 101). The dosage of copper hydroxide was based on recommendations of the manufacturer to field application for the bacterial spot. Culture tubes were placed on a shaking incubator at 28°C and incubated at 200 rpm for 4 hr. Aliquots (0.1 ml) of appropriate dilutions then were plated on triplicate plates of YDC medium, evenly spread with a sterile L-shaped glass rod, and incubated at 28°C for 48 hr. Mixtures of bacterial suspensions and buffered saline served as controls.

Four strains, HN94-1 and HP93-2 (sensitive) and HN94-3 and HN94-5 (resistant), were tested for viability in the several different copper sulfate solutions after 4 hr of exposure. The several concentrations of copper sulfate solution were prepared from 512 $\mu\text{g}/\text{ml}$ to 1 $\mu\text{g}/\text{ml}$ of copper in diluted water.

RESULTS

Identification of the pathogen. Bacterial strains identified as *X. campestris* pv. *vesicatoria* showed many typical characteristics of xanthomonads. The bacteria were yellow, rod-shaped, aerobic, Gram-negative, oxidase-negative and catalase-positive, and utilized glucose, arabinose, mannose, trehalose, and cellobiose for acid production. The strains did not utilize asparagine as a sole source of carbon and nitrogen, and failed to reduce nitrates. All bacterial strains were pathogenic to the pepper cultivar ECW. Based on biochemical, nutritional, and pathogenicity tests, it was confirmed that all bacterial strains isolated belonged to *X. campestris* pv. *vesicatoria*.

Table 2. Race and sensitivity to copper sulfate (200 $\mu\text{g}/\text{ml}$) of *Xanthomonas campestris* pv. *vesicatoria* isolated from pepper plants in Korea in 1993–1994

Province	County	No. of strains tested	No. of strains			
			Race 1		Race 3	
			Cu ^R ^a	Cu ^S	Cu ^R	Cu ^S
Kangwon-do	Wonju	4	0	1	0	3
Kyunggi-do	Anseong	1	0	0	0	1
	Hwaseong	3	0	2	0	1
	Icheon	4	0	1	0	3
	Suwon	1	0	0	0	1
Kyungsang-do	Yeoju	3	0	0	0	3
	Andong	2	0	1	0	1
	Changryung	1	0	0	0	1
	Ponghwa	1	0	0	0	1
	Youngcheon	2	0	1	0	1
	Yecheon	4	0	0	0	4
Cheolla-do	Cheongeu	6	0	4	0	2
	Hampyung	2	0	0	0	2
	Kimje	3	0	0	0	3
	Youngkwang	6	0	1	0	5
Cheju-do	Namcheju	1	0	0	0	1
Chungcheong-do	Asan	2	0	1	0	1
	Cheonan	6	5 ^b	0	0	1
	Choongwon	1	0	0	0	1
	Eumseong	2	0	1	0	1
	Jincheon	3	0	1	0	2
	Okcheon	2	0	2	0	0
	Poun	1	0	0	0	1
	Youngdong	1	0	0	0	1
	Yeongi	4	0	3	0	1
	Total		66	5	19	0

^a Cu^R: copper-resistant strain, Cu^S: copper-sensitive strain.

^b The HN 94-4 strain, which was isolated from Cheonan county, was resistant to copper sulfate (200 $\mu\text{g}/\text{ml}$) and streptomycin sulfate (100 $\mu\text{g}/\text{ml}$).

Race determination. Of the total 66 strains of *X. campestris* pv. *vesicatoria* tested, 42 strains were race 3 and 24 strains were race 1, while race 2 was not detected (Table 2). The distribution of races shows that race 3 is prevailing in Korea.

Sensitivity to copper and streptomycin. Five of all 66 strains tested grew on SPA medium amended with 200 µg/ml of copper sulfate and were therefore considered resistant to copper. Only one strain, HN 94-

4 was resistant to streptomycin sulfate (100 µg/ml). Five out of 24 race 1 strains were resistant to copper but none of race 3 was resistant to copper (Table 2).

The 4-hr exposure to copper hydroxide confirmed the results of the agar plate assay. The sensitive strain, HN 94-1 was completely killed. On the other hand, resistant strains survived more than 1×10^6 cfu/ml in culture tubes containing copper (Table 3).

One µg/ml of copper sulfate in solution killed all cells of the sensitive strains or reduced to very low population levels in 4 hr of exposure, but the viability of resistant strains appeared undiminished even in 128 µg/ml when compared with the control. Some reduction in the viability of the resistant strains occurred in 256 µg/ml of copper. All cells were killed in 512 µg/ml of copper during 4 hr of exposure regardless of sensitive and resistant bacterial strains (Table 4).

DISCUSSION

Copper resistance in *X. campestris* pv. *vesicatoria* has been reported in California (6), Florida (14), North Carolina (18), Oklahoma (3), Barbados (28), Italy (4), and Mexico (1). This is the first report concerning the copper resistance in *X. campestris* pv. *vesicatoria* in Korea. Copper-resistant strains of *X. campestris* pv. *vesicatoria* were isolated from only one location in Cheonan county, but no copper-resistant strain was detected in other counties (Table 1). In Cheonan county those plots have been applied with copper hydroxide re-

Table 3. Quantitative assay of effect of exposure to copper hydroxide *in vitro* on populations of representative strains of *Xanthomonas campestris* pv. *vesicatoria* varying in sensitivity to copper^a

Strain	Sensitivity classification ^b	Population after 4 hr (cfu/ml)
HN 94-1 ^c	Sensitive	0
HN 94-2	Resistant	5.14×10^7
HN 94-3	Resistant	4.54×10^7
HN 94-4	Resistant	4.04×10^7
HN 94-5	Resistant	4.29×10^7
HN 94-6	Resistant	4.94×10^7
71-21 (control)	Sensitive	0
07882 (control)	Resistant	5.84×10^7

^a Suspension (5 ml) of bacteria in sterile, buffered saline at initial populations of 2.0×10^8 cfu/ml exposed to 5 ml of a 2 g/l suspension of commercial copper hydroxide bactericide on a shaking incubator for 4 hr. Data are averages of three replications.

^b Based on qualitative assay.

^c HN 94 strains were isolated from Cheonan county.

Table 4. Viability of four strains of *Xanthomonas campestris* pv. *vesicatoria* after exposure to various amounts of soluble copper from copper sulfate for 4 hr

Copper (µg/ml)	Copper-sensitive strains			Copper-resistant strains		
	HN-94-1	HP 93-2 ^a	71-21 (control)	HN 94-3	HN 94-5	07882 (control)
0	+ ^b	+	+	+	+	+
1	-	±	±	+	+	+
2	-	-	-	+	+	+
4	-	-	-	+	+	+
8	-	-	-	+	+	+
16	-	-	-	+	+	+
32	-	-	-	+	+	+
64	-	-	-	+	+	+
128	-	-	-	+	+	+
256	-	-	-	-	±	-
512	-	-	-	-	-	-

^a HP 93-2 strain was isolated from Hampyung county.

^b + : Growth on sucrose peptone agar (SPA), ± : growth on SPA with fewer colonies, - : no growth on SPA. Each + or - represents three replicates.

peatedly. In this study, all copper-resistant strains were belong to race 1. This result was similar to those reported by Ritchie and Dittapongpitch (18) in 1991 and Bounaurio *et al.* (4) in 1994 that race 2 and some race 1 strains were copper-resistant. It has been demonstrated that copper resistance in race 2 is encoded by a gene cluster situated in a self-transmissible plasmid, which in turn is linked to the avirulence gene *avrBs1* that determines race 2 (22). However, it has not been known whether copper resistance in copper-resistant race 1 is genetically similar to that of race 2 strains. The absence of copper resistance in race 3 observed in this study supported the results of Ritchie and Dittapongpitch (18) in 1991 and Bounaurio *et al.* (4) in 1994.

Streptomycin resistance in this pathogen was observed in the 1950s (26). In this study, only one strain was streptomycin-resistant. This streptomycin-resistant strain was also copper-resistant, but copper-resistant strains were not always streptomycin-resistant. In *Staphylococcus aureus* Rosenbach, resistance to the metal cadmium is linked to penicillin and mercury resistance (21).

The relatively high frequency of occurrence of this pathogen and the detection of copper- and streptomycin-resistant strains give a negative impact on disease management strategies and indicate the importance of having pathogen-free seeds and transplants. The occurrence of copper and streptomycin resistance is further compounded by the possible loss of the use of maneb and mancozeb, which enhance the effectiveness of copper for the management of both copper-sensitive and especially -resistant strains of this pathogen (9, 14).

Nam *et al.* (16) reported that all the strains collected in Korea during the period from 1985 to 1986 were race 1, and in 1990 Kim *et al.* (13) reported that race 3 as well as race 1 was widely spread in Korea. In this study pepper races 1 and 3 of *X. campestris* pv. *vesicatoria* were detected but race 2 was not found in Korea. Race 3 was prevalent, representing 64% of the strains obtained for two years.

The ecology of the strains of *X. campestris* pv. *vesicatoria* is of interest. Race 2 of the pepper strain was found very seldom outside Florida, but race 1 occurred in Florida and in other areas of the world (5). This is the evidence that race 2, at least, was endemic to Florida. The endemic nature of the bacterium and the wise use of copper to control the bacterial spot disease

would be conducive to selecting and maintaining a copper-resistant strain. The copper-sensitive strains may be imported into Florida on contaminated seeds. As reported here, races 1 and 3 of this pathogen have the potential to occur in any pepper-growing area where the environment is favorable for bacterial spot. If bacterial spot is to be reliably controlled by host resistance, cultivars with multiple genes for resistance are essential. The use and deployment of resistant cultivars to all three races of *X. campestris* pv. *vesicatoria*. may provide the best disease management strategy.

요 약

1993년과 1994년에 걸쳐 한국의 고추 재배 지역에서 분리한 고추 더태이병균은 66개의 균주중 24개가 race 1이였으며, 나머지 42 균주가 race 3이였다. Race 2 균주는 발견되지 않았다. 총 66개의 균주중 8%가 200 µg/ml의 황산구리에 저항성을 보였으며, 100 µg/ml의 streptomycin sulfate에는 단지 한 균주만이 저항성을 나타냈다. Race 1 균주중 21%가 구리에 저항성을 보였으나 race 3 균주는 모두 구리에 감수성을 나타냈다. 모든 감수성 균주는 구리 농도 1 µg/ml에서 죽거나 아주 낮은 수준만이 생존하였으나 저항성 균주는 128 µg/ml에서도 생존하였다.

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