CHARACTERIZATION OF *PSEUDOMONAS SYRINGAE* pv. *SYRINGAE* ISOLATED FROM MANGO IN SICILY AND OCCURRENCE OF COPPER-RESISTANT STRAINS

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SUMMARY

Mango (*Mangifera indica*) is grown throughout a wide range of frost-free climates and is one of the world's most important fruit crops. During 2010-2014, severe symptoms of bacterial apical necrosis (BAN) caused by *Pseudomonas syringae* pv. *syringae* (*Pss)* were observed on mango plants throughout all mango-growing areas in Sicily (southern Italy). The causal agent was identified based on phenotypic, genotypic and pathogenicity tests. The genetic variability of strains obtained from different areas and cultivars were assessed by rep-PCR and allowed clear differentiation of the *Pss* strains isolated from mango from other representative strains of the pathovar. In addition, the respective production of syringomycin, syringopeptin and mangotoxin was checked by *in vitro* tests and PCR detection. All tested strains showed the presence of *mgoA* and *mgoB* of the mangotoxin operon. Some copper-resistant *Pss* strains showing *cusCBA* genes were also found in some orchards. This feature could explain the failure of the disease control using copper compounds. Among 71 strains tested using *in vitro* assay, no copper-sensitive strains were detected, whereas 44 strains (62%) had high resistance, 16 strains (22.5%) were resistant and 11 strains (15.5%) had low resistance to copper sulfate. To our knowledge, this is the first report of copper resistance among *Pss* strains causing BAN on mango in Sicily.

Key words: Mangifera indica, apical necrosis, mangotoxin**,** pathogenicity, copper resistance.

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INTRODUCTION

Mango (*Mangifera indica* L., family Anacardiaceae) is grown throughout a wide range of frost-free climates and is one of the most important fruit crops in the world (Litz, 1998). There are about 100 recorded mango-producing countries (FAOSTAT, 2013). The first commercial mango orchard in Italy was planted during 1980-1990 in the Catania province (Sicily). Thereafter, its cultivation expanded to the other provinces of Sicily (Messina, Ragusa and Palermo) and to the neighboring Calabria region. In these regions, the most commonly grown cultivars are Kensington Pride, Tommy Atkins, Osteen, R2E2, Maya, Kent, Irwin, and Keitt.

In Italy the commercial viability of this crop is threatened by recently reported fungal (Ismail *et al*., 2013a, 2013b; Ahmed *et al*., 2014; Ismail *et al*., 2015) and bacterial diseases. Bacterial apical necrosis (BAN), caused by *Pseudomonas syringae* pv. *syringae* (*Pss*) is an important bacterial disease of mango crops in the Mediterranean area (Pinkas *et al*., 1996; Cazorla *et al*., 1998; Torta *et al*., 2003) and in subtropical areas, such as northwestern Australia (Golzar and Cother, 2008). The disease is characterized by rapidly expanding necrotic lesions on leaves, buds, stems and floral panicles, whereas fruits are not affected. The symptoms develop more strongly in cool years, and the severity of the outbreaks depends to a great extent on winter and spring temperatures, on rain or dew which is essential for inoculum dissemination to other buds and leaves, and wind that can facilitate disease development by causing micro injuries through which the pathogen can penetrate (Cazorla *et al*., 1998).

BAN disease significantly reduces viability of mango crops, causing severe yield reductions and economic losses mainly in years with severe attacks. Treatment with copper compounds, mostly Bordeaux mixture, are routinely applied during autumn-winter to reduce the number of cells of *Pss* and prevent infections of tree crops (Cazorla *et al*., 2002). However, in Spain copper tolerance has been reported among *Pss* strains (Cazorla *et al*., 2002), and consequently the efficacy of copper has been unsatisfactory in many cases.

Fig. 1. Typical symptoms of bacterial apical necrosis on mango trees. Diseased tree presenting a generalized blast (a), blight of shoots (b), initial symptoms of necrosis along the central vein of mango leaves extending to the leaf petioles and stem (c) and detail of affected mango stems and associated drop of exudates (d).

Recently, there were heavy BAN infections of new mango orchards in several Sicilian areas, and surveys were carried out to determine whether or not the disease was also present in the new cropping regions. Indirect evidence (i.e. failure to effectively control the disease) indicated that *Pss* strains in Sicily are resistant to copper compounds routinely used by growers. Therefore, a study was also carried out to determine if copper-resistant *Pss* strains are present in various mango orchards throughout Sicily.

This paper reports the isolation, identification and characterization of bacterial strains isolated from diseased tissues of different mango cultivars in Sicily, their genetic typing by rep-PCR, the presence of the mangotoxin operon and the occurrence of copper-resistant *Pss* strains.

MATERIALS AND METHODS

Isolation of bacterial strains. During winter and early spring of 2010-2011 and 2013-2014, commercial mango orchards planted with cvs Kensington Pride, Tommy Atkins and Osteen were surveyed in Sicily (southern Italy) in seven locations (one orchard in the province of Palermo, three orchards in Messina and three in Catania). Samples with necrotic symptoms (Fig. 1) were randomly collected (six samples per orchard). Affected leaves, petioles and buds were cut, placed in sterile plastic bags, transported to the laboratory, and processed on the day of sampling or stored at 5°C.

The samples were processed by two methods: (i) plant materials were surface-disinfected by immersion in 10% bleach solution (0.5% sodium hypochlorite) for 1 min, then rinsed in sterile distilled water. Small pieces between healthy and infected tissues (2-4 mm^2) were plated on King medium B (KB) (King *et al*., 1954) and nutrient agar (NA) (Oxoid, USA); (ii) Plant materials (5 g) were placed in 100 ml of sterile 0.1 M potassium phosphate buffer (PPB) (pH 7.0) containing 0.1% bactopeptone, sonicated in an ultrasonic cleaner (Brasonic 52, Branson Cleaning Equipment, USA) for 7 min, and tenfold dilutions (100 µl) were spiral plated onto KB and NA using a Spiral Plater Eddy Jet (IUL Instruments, Spain). Plates were incubated at 26 ± 1°C for 72 h. Characteristic colonies were re-streaked to ensure purity. Bacteria were routinely cultured on KB

agar and stored at −80°C in 1.5 ml of nutrient broth and 15% glycerol.

Identification of bacterial strains. Fluorescent bacterial colonies were consistently isolated from the buds, leaf petioles and leaf tissues adjacent to midribs and intervenial lesions. Fluorescent bacteria with positive hypersensitivity on tobacco (*Nicotiana glutinosa* L.) were identified according to biochemical tests previously described (Lelliott *et al*., 1966; Lelliott and Stead, 1987; Braun-Kiewnick and Sands, 2001), including LOPAT tests (levan production, oxidase reaction, potato soft rot and arginine dihydrolase), oxidative metabolism of glucose, hydrolysis of esculin and gelatin, and utilization of glucose, mannitol, sorbitol, gluconate, propionate, L-lactate, L-histidine, inositol and adonitol. All inoculated plates or tubes, in duplicate, were incubated at 25° for 3-5 days. Tests were performed twice.

The Biolog identification system (Biolog MicroLog 4.0 System, Biolog, USA) was also used to characterize 24 strains, representative of the seven orchards and the three cultivars. Tests were performed according to the manufacturer's instructions. The *Pss* strain B728a and *P. fluorescens* strain A506 were used as controls.

The list of 71 *P. syringae* strains belonging to LOPAT group 1A isolated from mango and of 24 *P. syringae* strains previously isolated from other crops is given in Table 1.

Pathogenicity tests. Pathogenicity tests were performed on young bean pods, immature lemons, tomato seedlings and mango leaves and buds. Bean pods and lemons were surface-disinfected (1 min in 0.5% sodium hypochlorite), rinsed in sterile distilled water, inoculated by placing a drop (20 µl) of a bacterial suspension (10^6 CFU ml^{-1} in PPB) on the surface and pricking through the drops using sterile needles as previously described (Cazorla *et al*., 1998; Scortichini *et al*., 2003; Cirvilleri *et al*., 2005a). Controls were treated with sterile PPB. After inoculation, bean pods and lemons were incubated in moist trays at 22 ± 1°C for 4-7 days. Pathogenic reaction was scored as positive given the presence of black necrotic lesions.

Four-week-old tomato seedlings, previously surface-disinfected as described above, were inoculated with a 20 µl drop of bacterial suspension (10^6 CFU ml^{-1} in PPB) deposited on fresh wounds made on the leaves. After inoculation, the plants were covered by transparent polyethylene bags for 48 h and placed in a growth chamber at 22 ± 1°C with a 16 h (light)/8 h (dark) photoperiod. Controls were treated with sterile PPB. Disease symptoms were checked after 7 days incubation. The leaves were scored as positive if necrotic areas and wilting were present. Assays were repeated three times for each strain, and each assay was carried out on five different fruits, pods or tomato leaves.

To test pathogenicity on mango, two methods were used: (i) young detached mango leaves (cv. Kensington Pride) were inoculated by placing 20 µl drops of bacterial suspension (10^6 CFU ml^{-1} in PPB) on freshly wounded leaf laminae and midribs. Each isolate was inoculated on three leaves. Controls were treated with sterile PPB. Inoculated leaves were incubated in moist trays at 22 ± 1°C and disease symptoms checked after 7-10 days. Pathogenic reactions were recorded as positive if necrotic areas around inoculation sites and progressive necrosis along the midribs and interveinal tissue were present. The assay was repeated twice; (ii) Buds of 1-year-old cv. Kensington Pride plants growing in pots were inoculated with 20 µl of a 10^6 CFU ml^{-1} bacterial suspension in PPB forcing the inoculum with a microsyringe. Each isolate was inoculated on three buds. Controls were treated with sterile PPB. Plants were covered and incubated as previously described. The occurrence of apical necrosis symptoms on inoculated buds was recorded after 30 days. The assay was repeated twice.

To confirm the presence of *Pss* in inoculated tissues, re-isolation was carried out from plant buds showing necrotic symptoms after 30 days. Each bud was surface-disinfected, rinsed in sterile distilled water, cut into small pieces and homogenized in sterile PPB. Tenfold dilutions were spiral-plated onto KB, and identity of bacterial isolates was verified by fluorescent pigment production and LOPAT tests, as described above.

Syringomycin and syringopeptin production. The production of syringomycin and syringopeptin by *Pss* strains was determined by the growth inhibition test on potato-dextrose agar (PDA) using *Rhodotorula pilimanae* and *Bacillus megaterium*. Isolates were grown on KB for 24 h, then suspended in sterile PPB and diluted to a concentration of 10^6 CFU ml^{-1}. Then 20 µl of a overnight *Pss* culture were spotted onto PDA, followed by incubation for 48 h at 26 ± 1°C. Subsequently, the plates were sprayed with an overnight culture of *R. pilimanae* or *B. megaterium* and incubated for 24-48 h at 26 ± 1°C. The presence of lipodepsipeptides was characterized by development of a zone of inhibition surrounding *Pss* colonies. The *Pss* strain 48SR2 and *P. fluorescens* strain A506 were used as controls.

Presence of *mgo* genes of the mangotoxin operon. The possible presence of the *mgoA* and *mgoB* genes, present in the *mgo* operon and governing the production of mangotoxin, was assayed in 24 *Pss* strains representative of mango cultivar, area of origin and year of isolation, according to the procedures of Carrión *et al.* (2014). These genes were also assessed in four of the *Pss* strains previously isolated from *C. sinensis* (Table 1).

Molecular characterization by rep-PCR. Twenty-four representative *Pss* strains isolated from mango were compared with other 24 representative *P. syringae* strains isolated from different hosts (see Table 1) by rep-PCR fingerprint analysis, using BOX, ERIC and REP primer sets (Louws *et al*., 1994). The assay was performed according to procedures previously described (Ferrante and Scortichini,

Table 1. Strains of *Pseudomonas syringae* pv. *syringae* used in this study.

Strains[x]	Host	Variety	Area of origin	Year	Patogenicity on	
					Bean[y]	Lemon[z]
PSM 45[abcd]; PSM 64[a]; PSM 70[a]	*Mangifera indica*	Kensington Pride	Catania (Italy)	2010	+	+
PSM 47[abcd]; PSM 60[abcd]; PSM 71[abcd]	"	"	"	2010	+	++
PSM 43[abcd]; PSM 44[a]; PSM 46[a]; PSM 49[a]; PSM 50[a]; PSM 52[a]	"	"	"	2011	+	++
PSM 48[abcd]	"	"	"	2011	++	+
PSM 51[abcd]; PSM 53[abcd]	"	"	"	2011	++	++
PSM 54[abcd]; PSM 57[abcd]; PSM 59[a]; PSM 69[abcd]	"	"	Messina (Italy)	2010	+	++
PSM 58[abcd]	"	"	"	2010	+	+
PSM 32[abcd]; PSM 33[abcd]; PSM 34[abcd]; PSM 35[xd]; PSM 36; PSM 37; PSM 38; PSM 39; PSM 40; PSM 42	"	"	"	2014	+	++
PSM 41	"	"	"	2014	+	+
PSM 55[abcd]; PSM 56[abcd]; PSM 66[ad]	"	"	Palermo (Italy)	2010	++	+
PSM 61[a]; PSM 72[a.]	"	"	"	2010	+	+
PSM 62[a]; PSM 65[a]	"	"	"	2010	+	-
PSM 63[abcd]	"	"	"	2010	+	++
PSM 20[a]; PSM 21[abcd], PSM 22[abcd], PSM 23[ad], PSM 25[ad], PSM 26[a], PSM 29[a]; PSM 30[a]; PSM 31[a]	"	Osteen	Catania (Italy)	2014	+	++
PSM 24[a]	"	"	"	2014	+	+
PSM 27[a]; PSM 28[a]	"	"	"	2014	+	+
PSM 1; PSM 2[abcd]; PSM 3[abcd]; PSM 4; PSM 5; PSM 6; PSM 7; PSM 8	"	Tommy Atkins	Messina (Italy)	2014	+	++
PSM 9[abcd]; PSM 10[abcd]; PSM 11; PSM 12; PSM 13; PSM 14; PSM 15; PSM 16; PSM 17; PSM 18; PSM 19	"	"	"	2014	+	++
PSM 68	"	"	"	2014	+	+
PSS PVCT 10.2 [c]; PSS PVCT 41 [c]; PSS PVCT 119[c]; PSS PVCT 281[c]; PSS PVCT 40SR4[c]; PSS PVCT 48SR2[c]	*Citrus sinensis*		Catania (Italy)	1990		
PSS AL513[c]	*Citrus sinensis*		USA (S.E. Lindow)			
PSS PVCT 23P[c]; PSS PVCT 76P[c]	*Pirus communis*		Catania (Italy)	1998		
PSS AID 122A[c]	*Prunus amygdalus*		Catania (Italy)	1988		
PSS PVCT 1.2S[c], PSS PVCT 1.4S[c]	*Strelitzia reginae*		Catania(Italy)	2000		
PSS PVCT 4[c]	*Cynara scolimus*		Catania (Italy)	1992		
PSS AID 48[c]; PSS AID 33[c]	*Fragaria×ananassa*		Catania (Italy)	1988		
PSS ISF 282[c]	*Castanea sativa*		Italy	1996		
PSS ISF304[c]; PSS ISF309[c]	*Triticum aestivum*		Italy	1996		
PSS ISF 310[c] (=NCPPB2612)	*Triticum aestivum*		New Zealand			
PSS ISF293[c] (=B359)	*Setaria italica*		Australia (J.E. DeVay)			
PSS ISF291[c] (=SY12)	*Syringa vulgaris*		Japan (D.C. Gross)			
PSS ISF286[c] (=Y37)	*Phaseolus vulgaris*		UK (G.L. Ercolani)			
PSS ISF 332[c]	*Phaseolus vulgaris*		Italy	1996		
PSS B728a[c]	*Phaseolus vulgaris*		USA (S.E. Lindow)	1986		

[a] mango strains used in pathogenicity tests on tomato leaves and mango leaves and buds.

[b] mango strains identified as *P. syringae.* pv. *syringae* by Biolog system and analyzed for *mgo* genes.

[c] *Pseudomonas syringae* strains characterized by repPCR.

[d] *Pseudomonas syringae* strains analyzed for *cusCBA* genes.

[x] PSM=*Pseudomonas syringae* from mango; PSS = *Pseudomonas syringae* from other hosts; AID: Agricultural Industrial Development, Catania, Italy; ISF: C.R.A. Istituto Sperimentale per la Frutticoltura, Roma, Italy; NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK; PVCT: Plant Pathology, University of Catania, Italy.

[y] +: necrotic lesions ; ++=necrotic lesions surrounded by reddish margin.

[z] +: necrotic lesions (1-2 mm); ++= necrotic lesions (3-5 mm).

2010, 2014). Briefly, genomic DNA was extracted with the GeneElute bacterial genomic DNA kit (Sigma Aldrich, USA). DNA concentrations were estimated by measuring absorbance at 260 and 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, USA). Amplicons were separated by electrophoresis on 2% (w/v) agarose gels in 0.5× TAE buffer at 50 V and 4°C for 16 h. Cluster analysis was performed on a similarity matrix, subjected to the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm and Dice's coefficient, using web tools of www.pubmlst.org.

***In vitro* copper resistance and *cusCBA* genes presence.** Resistance to copper was determined using MGY media (Bender and Cooksey, 1986). Copper from a filter-sterilized (0.45 µ) stock solution of 160 mM $CuSO_4 \cdot 5H_2O$ (Merck, USA) was added to autoclaved media cooled to 50°C immediately before pouring to achieve the desired

copper concentration. To measure the minimum concentration of copper that prevents colony growth (minimum inhibitory concentration, MIC), bacterial suspensions were adjusted to approximately 10^5 CFU ml^{-1} in distilled water, and a 10 µl drop of each suspension was placed on plates of MGY media (four spots per plate) containing copper of 0-3.2 mM. Cultures were incubated for 96 h at 26 ± 1°C, and MIC was recorded. Strains were considered sensitive if they grew only on media amended with 0.40-0.80 mM of cupric sulfate, as of low resistance with 1.0-1.6 mM, as resistant with 1.8-2.4 mM and highly resistant with 2.6-3.2 mM. Control cultures of all isolates were incubated in similar conditions in non-amended MGY for comparison purposes. A copper-resistant and a copper-sensitive strain, *P. syringae* Al513 and *P. syringae* Al417, respectively (Rogers *et al.*, 1994), were used as controls. Each test was repeated twice with three replications per copper concentration.

The presence of *cusCBA* genes in 28 *Pss* representative strains was checked by a PCR-based assay. Primers were designed using Primer3 software: they were based on the *P. syringae* pv. *syringae* UMAF0081 sequence deposited at NCBI databank (accession No. JX645720) (Gutierrez-Barranquero *et al.*, 2013b). The primer list is reported in Table 3. All PCR reactions were performed in a Bio-Rad Mini thermal cycler with the following cycling conditions: denaturation at 95°C for 5 min; 35 cycles of 35 sec of annealing at 58°C; and extension at 72°C for 1 min; and 5 min of final extension at 72°C. Amplification products were separated in 1% agarose gels at 50 V and 4°C for 16 h, and visualized by a Bio-Rad Gel Logic 100 UV transilluminator.

RESULTS

Isolation and identification of bacterial strains from mango orchards. Isolations from mango tissues showing typical BAN symptoms yielded 71 fluorescent bacterial colonies that were selected and purified on KB and NA (Table 1).

Fluorescent colonies on KB were levan positive, oxidase and arginine dihydrolase negative, potato soft-rot negative and tobacco hypersensitivity positive (LOPAT tests group Ia). Moreover, all isolates showed oxidative metabolism of glucose, hydrolyzed esculin and gelatin, and utilized glucose, mannitol, inositol, sorbitol, gluconate, propionate, L-lactate and L-histidine, but not adonitol. Twenty-four representative isolates (Table 1) characterized using the Biolog system were identified as *Pss* with a probability range of 90-94%.

All isolates were considered as putative *Pss* and were further assessed for pathogenicity, virulence, copper resistance and *cusCBA* genes, syringomycin, syringopeptin and mangotoxin production and molecularly typed by rep-PCR.

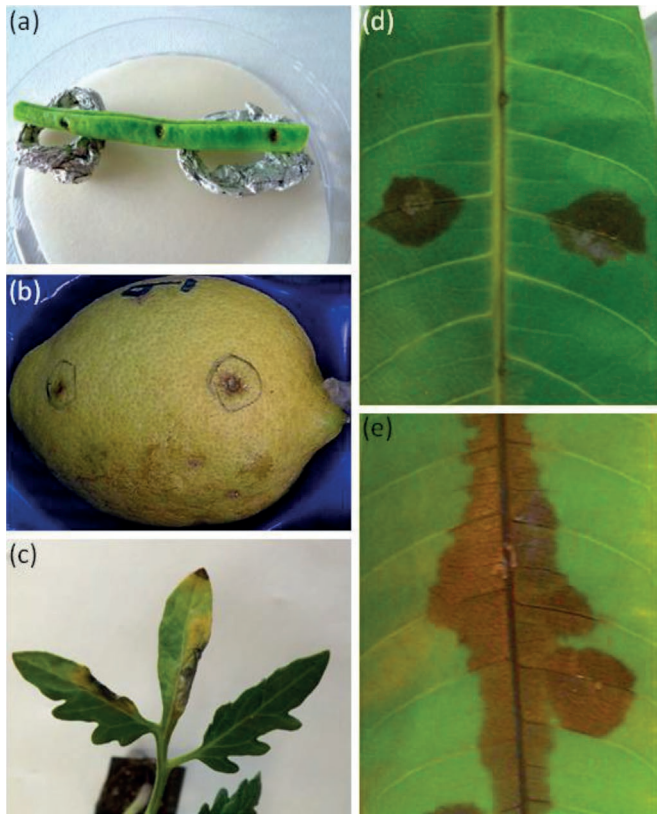

Fig. 2. Symptoms induced by inoculation with *Pseudomonas syringae* pv. *syringae.* Black, necrotic lesions on bean (a) and lemon (b), necrotic symptoms in tomato leaves (c), necrotic spots surrounding the inoculation points on mango leaf (d) and necrosis of midrib and intervein tissues on mango leaf (e).

Pathogenicity tests. The isolates caused dark sunken spots on bean pods, sometimes surrounded by a reddish margin (Fig. 2a), and deep black, necrotic lesions on lemon fruits in a size range of 1-5 mm (Fig. 2b). Tomato leaves showed necrotic areas and leaf wilting 7 d after inoculation (Fig. 2c). Symptoms described above were similar to the symptoms previously reported on differential hosts in response to *Pss* strains isolated from other crops (Cazorla *et al.*, 1998; Scortichini *et al.*, 2003; Cirvilleri *et al.*, 2005a).

The *Pss* strains inoculated on mango leaves produced observable disease symptoms consisting of necrotic spots (about 10 mm in diameter) surrounding the bacterial inoculation point (Fig. 2d). The same strains produced necrotic lesions of central vein and interveinal tissues after midrib inoculation (Fig. 2e).

The *Pss* strains individually inoculated onto healthy buds and shoots of mango plants produced typical symptoms of apical necrosis (Fig. 3). Symptoms were observed after 30 days (Fig. 3b), and the first symptoms were observed 7 days post inoculation (Fig. 3a). In sterile PPB-treated controls, there were no necrotic lesions around the inoculation sites. To confirm the presence of *Pss* in inoculated tissues, reisolation was carried out from plant buds showing necrotic symptoms, which yielded fluorescent

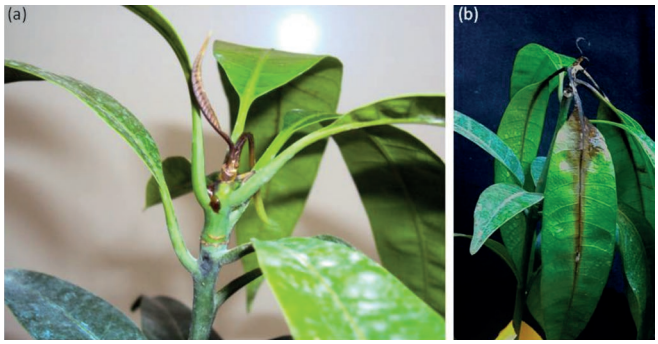

Fig. 3. Buds of cv. Kensington Pride artificially inoculated with strains of *Pseudomonas syringae* pv. *syringae* isolated from mango. Bud necrosis and drop of exudates 1 week (a) and 4 weeks (b) after inoculation.

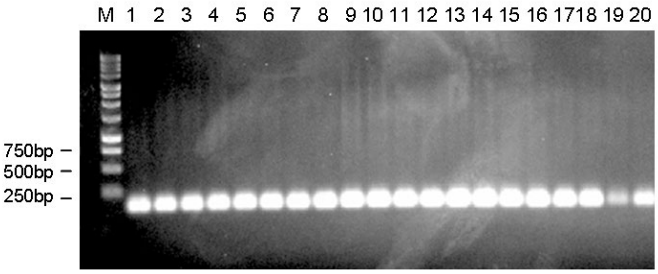

Fig. 4. Representative gel for the detection of gene fragments of mangotoxin in *Pseudomonas syringae* pv. *syringae* strains isolated from mango in Italy during 2010-2014 (lanes 5-20), and in *P. syringae* pv. *syringae* strains from different hosts (lanes 1-4). M: molecular size marker 1-kb DNA ladder (Promega). Lane 1: Pss10.2; lane 2: $Pss41_2$; lane 3: 119_2; lane 4: Pss 281_1; lane 5: PSM9; lane 6: PSM10; lane 7: PSM21; lane 8: PSM22; lane 9: PSM33; lane 10: PSM34; lane 11: PSM43; lane 12: PSM47; lane 13: PSM48; lane 14: PSM51; lane 15: PSM53; lane 16: PSM55; lane 17: PSM56; lane 18: PSM60; lane 19: PSM69; lane 20: PSM63.

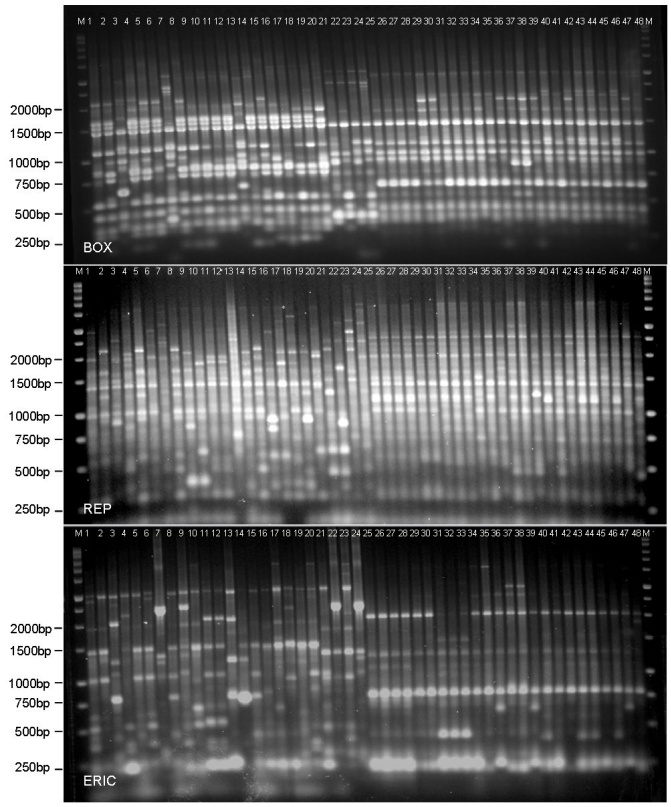

Fig. 5. Representative repetitive-sequence PCR fingerprint patterns for genomic DNAs of *Pseudomonas syringae* pv. *syringae* strains isolated from mango during 2010-2014 in various locations in Sicily (lanes 25-48) by using REP, ERIC and BOX primer sets. The strains were compared with *P. syringae* pv. *syringae* strains previously isolated in Italy from different hosts (lanes 1-24). M: molecular size marker 1-kb DNA ladder (Promega) (see also Table 1). Lane 1: Pss10.2, lane 2: Pss41, lane 3: Pss119, lane 4: Pss281, lane 5: Pss40SR4, lane 6: Pss48SR2, lane 7: PssA1513, lane 8: Pss23P, lane 9: Pss76P, lane 10: PssISF282, lane 11: Pss1.2S, lane 12: Pss1.4S, lane 13: PssAID122A, lane 14: PssAID48, lane 15: PssAID33, lane 16: PssISF304, lane 17: PssISF309, lane 18: PssISF310, lane 19: PssISF293, lane 20: PssISF291, lane 21: PssB728a, lane 22: PssISF332, lane 23: PssISF286, lane 24: Pss4, lane 25: PSM2, lane 26: PSM3, lane 27: PSM9, lane 28: PSM10, lane 29: PSM21, lane 30: PSM22, lane 31: PSM32, lane 32: PSM33, lane 33: PSM34, lane 34: PSM43, lane 35: PSM45, lane 36: PSM47, lane 37: PSM48, lane 38: PSM51, lane 39: PSM53, lane 40: PSM54, lane 41: PSM55, lane 42: PSM56, lane 43: PSM57, lane 44: PSM58, lane 45: PSM60, lane 46: PSM69, lane 47: PSM71, lane 48: PSM63.

bacteria with phenotypic characteristics identical to those of the inoculated isolates.

Syringomycin and syringopeptin production. All the 71 strains tested produced syringomycin and syringopeptin as shown by the inhibition halo of *R. pilimanae* (7-18 mm), and of *B. megaterium* (3-15 mm).

Presence of *mgo* genes of the mangotoxin operon. Twentyfour representative *Pss* strains recovered from different mango cultivars in Sicily possessed the *mgoA* and *mgoB* genes of the mangotoxin operon (representative gel in Fig. 4). The presence of *mgoA* and *mgoB* was also detected in four *Pss* strains isolated also Sicily from *C. sinensis* (Fig. 4).

Repetitive-sequence PCR (rep-PCR) assay. The rep-PCR performed with ERIC, BOX and REP primer sets allowed clear differentiation of the 24 representative *Pss* strains isolated in Sicily from different cultivars of mango from other *Pss* strains obtained from different crops. Representative gels are shown in Fig. 5, and the corresponding dendrogram of strain relationships inferred by UPGMA is shown in Fig. 6. Variability among the *Pss* strains isolated from mango trees in Sicily was found with any primer used in the range of 250-2,000 bp, even though they showed an overall homogeneous pattern. Notably, the bacterial strains from Sicilian mangoes clustered separately from the *Pss* strains isolated from *Syringa vulgaris*, *Citrus sinensis*, *Pyrus communis*, *Prunus*

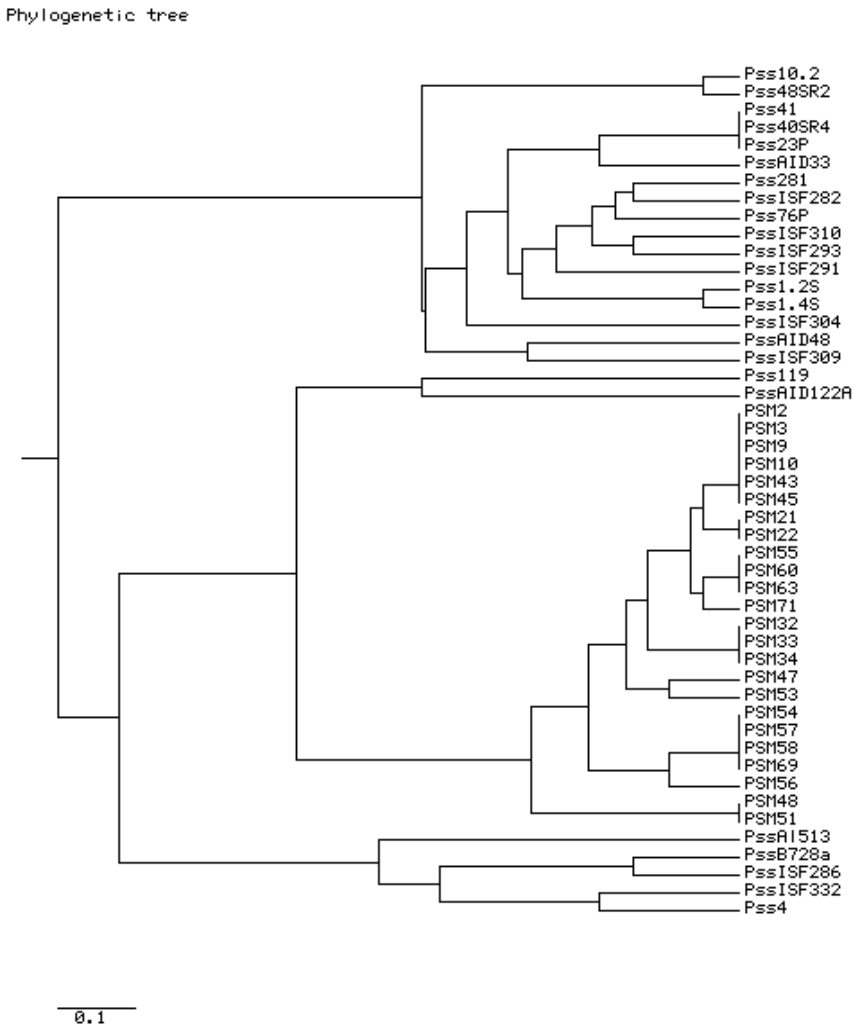

Fig. 6. Dendrogram of genetic relationships of the rep-PCR fingerprint relationships generated by 24 *Pseudomonas syringae* pv. *syringae* strains isolated from mango showing bacterial apical necrosis and by 24 *P. syringae* pv. *syringae* strains isolated from different hosts. Cluster analysis was performed using UPGMA and Dice's coefficients.

amygdali, *Castanea sativa*, *Strelizia reginae*, *Cynara scolymus*, *Fragaria×ananassa*, *Triticum aestivum*, *Setaria italica* and *Phaseolus vulgaris.* These *Pss* strains also showed a larger variability in the rep-PCR fingerprints.

***In vitro* copper resistance and *cusCBA* genes presence.** The *Pss* strains from Sicily exhibited variable levels of tolerance to copper ions added to MGY medium. Among 71 strains tested, 44 were highly resistant with MIC values of 2.6-3.2 mM of cupric sulfate (Table 2), 16 strains tolerated 1.8-2.4 mM and were considered resistant, and 11 able to grow only on 1.0-1.6 mM were considered of low resistance. Sensitive strains were not detected (Table 2).

Classification of data by cultivar revealed that in general *Pss* strains with high resistance to copper (>2.6 mM) were recovered in most orchards and all mango cultivars (21, 14 and nine strains, respectively, from cvs Kensington Pride, Tommy Atkins and Osteen), whereas the 11 low-resistance strains (1.0-1.6 mM) were collected only from cv. Kensington Pride.

The presence of *cusCBA* genes was detected in three out of 28 representative *Pss* strains namely PSM47, PSM60 and PSM63, that in *in vitro* test were characterized by high resistance to copper. These strains yielded PCR products of the expected size, 486 bp, 418 bp and 476 bp for *cusA*, *cusB* and *cusC*, respectively. A representative gel is shown in Fig. 7.

DISCUSSION

Biochemical characterization and results of LOPAT allowed the identification of 71 isolates as *P. syringae.* BIOLOG applied to 24 representative *P. syringae* strains allowed their identification as *Pss.* The surveys performed during winter and early spring in 2010/2011 and 2013/2014 in mango orchards in Sicily showed that *Pss*, the causal agent of BAN of mango, is widely distributed in all provinces sampled, both in the eastern and western areas of cultivation, including the Palermo province where the disease was first recorded (Torta *et al.*, 2003).

Table 2. Number of *Pseudomonas syringae* pv. *syringae* strains isolated from mango trees belonging to different categories of MIC values for cupric sulfate (mM) on MGY medium.

Place and year of isolation	Cultivar	MIC (mM) interval for cupric sulfate				
		Sensitive	Resistant			
		0.4-0.8	1.0-1.6	1.8-2.4	2.6-3.2	Total
Palermo (Balestrate) 2010	Kensington pride	-	-	8	-	8
Messina (Milazzo) 2010	Kensington pride	-	-	-	6	6
Messina (Caronia) 2014	Kensington pride	-	11	-	-	11
Messina (Caronia) 2014	Tommy Atkins	-	-	5	14	19
Catania (Fiumefreddo) 2010	Kensington pride	-	-	-	7	7
Catania (Acireale) 2011	Kensington pride	-	-	-	8	8
Catania (Carrubba) 2014	Osteen	-	-	3	9	12
Total		**-**	**11**	**16**	**44**	**71**

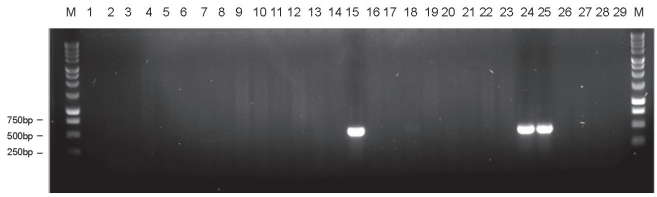

Fig. 7. Representative gel showing the occurrence *of cusA* gene in three *Pseudomonas syringae* pv. *syringae* strains isolated from mango trees in Sicily. M: molecular size marker 1-kb DNA ladder (Promega). Lane 1: PSM2, lane 2: PSM3, lane 3: PSM9, lane 4: PSM10, lane 5: PSM21, lane 6: PSM22, lane 7: PSM23, lane 8: PSM25, lane 9: PSM32, lane 10: PSM33, lane 11: PSM34, lane 12: PSM35, lane 13: PSM43, lane 14: PSM45, lane 15: PSM47, lane 16: PSM48, lane 17: PSM51, lane 18: PSM53, lane 19: PSM54, lane 20: PSM55, lane 21: PSM56, lane 22: PSM57, lane 23: PSM58, lane 24: PSM60, lane 25: PSM63, lane 26: PSM69, lane 27: PSM71, lane 28: PSM66, lane 29: negative control.

Previous studies, using both rep-PCR and MLST analysis, on *Pss* strains isolated from mangoes in Spain, showed the distinctiveness of such strains from *Pss* strains from other crops (Gutierréz-Barranquero *et al*., 2013a). Also in the present study, the *Pss* strains isolated from Sicilian mangoes clustered separately from the other *Pss* strains used for comparison. The possibility that strains from mango represent a tight and well-characterized cluster within *Pss* seems plausible. Rep-PCR typing, a low-cost analysis, confirmed its robustness and reliability in effectively distinguishing closely related strains (Cirvilleri *et al*., 1998, 2005a, 2005b; Scortichini *et al*., 1998; Natalini *et al*., 2006; Kaluzna *et al*., 2010; Gutierréz-Barranquero *et al*., 2013a).

As previously observed in Spain (Carrion *et al*., 2014), the present study revealed the presence of *mgoA* and *mgoB* genes of the mangotoxin operon that were found in all the representative *Pss* strains from mango.

Copper-resistant *Pss* strains were widely distributed on symptomatic mango leaves and shoots examined, and were detected during the survey in different orchards, showing that they can survive from season-to-season in the presence of copper applications. It is noteworthy that no strain sensitive to copper was detected, and that some *Pss* strains were tolerant to only low $CuSO_4$ concentrations (1.0-1.6 mM), whereas the majority were tolerant to much higher concentrations. Up to 62% of the *Pss* strains could tolerate >2.6 mM of $CuSO_4$ and were widely distributed over the years of sampling, areas and cultivars. The periodic selection pressure imposed by copper spray applications seemed to favor a widespread establishment of copper-tolerant bacterial populations, and might provide a reservoir of copper-resistance genes that could be acquired by copper-sensitive pathogens, as suggested by Cazorla *et al*. (2002). However, this article provides the first data on occurrence of copper-resistance in *Pss* population in Italy.

The presence of *cusCBA* efflux system genes (Rensing and Grass, 2003) and their relationship with copper resistance has recently been described in two *P. syringae* strains from different hosts (mango and cherry) and countries (Spain and USA) as well as in the draft genomes of *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci* (Gutierréz-Barranquero *et al*., 2013b). *CusCBA* genes were associated with copper resistance corresponding to 1.8 mM $CuSO_4$, whereas they were not found in copper-resistant strains showing lower MICs values (0.8-1.2 mM). The same authors (Gutierréz-Barranquero *et al*., 2013a) analyzed a collection of *Pss* strains from mango in southern Spain revealing the presence of 62 kb plasmids and suggesting their possible relationship with copper resistance. In their study, however, several copper-resistant strains were plasmid-less, and part of the copper-sensitive strains contained the plasmids.

In the present work we detected for the first time that *cusCBA* genes are also present in three copper resistant *Pss* strains from mango cultivars in Sicily. Also in this case the presence of the *cusCBA* genes was only associated with the highest resistant level to copper, higher (2.8-3.2) than those reported by Gutierréz-Barranquero *et al*. (2013b).

Recent studies have shown that several metals, including copper, can activate the production of siderophores by bacteria, thereby implicating siderophores in heavy metal tolerance (Schalk *et al*., 2011). Pyoverdine and pyochelin, the major siderophores produced by *P. aeruginosa*, are able to chelate several metals including Cu^{2+} (Braud *et*

Table 3. Primers used to detect *cusCBA* genes in *Pseudomonas syringae* pv. *syringae* strains isolated from mango trees in Sicily.

Primer name	Primer sequence	Amplicon size (bp)
cusA-F	5'-ATCTGCCACCATCGATAGGG-3'	486
cusA-R	5'-CCTGAAAAGCTGATCGAGGC-3'	
cusB-F	5'-CGATAGTTTGTACGGCAGCC-3'	418
cusB-R	5'-CCAAAATACGCCGATGAGCA-3'	
cusC-F	5'-ATGCGCGAGAGGTTCTTTTC-3'	476
cusC-R	5'-CAGCTCAACCTACAACGCTC-3'	

al., 2009) thus sequestering these metals in the extracellular medium outside the bacteria, preventing its diffusion across the bacterial membranes into the cells (Braud *et al*., 2010), and allowing bacteria to be more resistant to copper. This new evidence of siderophore implication in copper resistance could help us to explain the "discrepancy" between the relevant occurrence of copper-resistance observed *in vitro* and the presence of *cusCBA* genes detected solely in three strains.

The aggressiveness of the pathogen detected in the fields under study and the critical situation for protecting crops in the absence of bactericidal alternatives to copper-based formulations could, in addition to other environmental and agronomic factors, limit the spread of mango cultivation in Sicily. The presence of resistance to copper at different levels among *Pss* strains could explain the incomplete control of BAN using copper compounds in open fields. The economic importance of bactericide resistance in plant pathogens is emphasized by the lack of alternative bactericides to replace ineffective compounds.

The efficacy of copper compounds, mostly Bordeaux mixture, applied during autumn-winter is often limited and has led to increased dosage and frequency of copper applications. This scenario is common to several bacterial diseases that have been repeatedly exposed to copper, in which the lack of control is usually related to the selection of copper-resistant strains, as observed for *Pss* mango orchards in Spain, where intensive copper spraying was used for disease control (Cazorla *et al*., 2002).

More generally, in order to contain disease spreading, it is important to follow different procedures: (i) healthy plants from nurseries that follow strict phytosanitary practices should be used, and infection-free mother plant plots should be established; (ii) cultivars tolerant/resistant to BAN should be identified (cvs Kensington Pride, Tommy Atkins, Lippens and Manzanillo are very sensitive, while cvs Keitt and Sensation are more resistant); (iii) the exclusive use of copper compounds in control strategies should be avoided as biocontrol agents, successfully used to reduce the population of several bacterial diseases, could be an alternative strategy applicable in the near future.

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