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Expression of pathogenicity and virulence related genes in *Pseudomonas syringae* pv. *syringae* under copper stress

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Abstract

Stone fruit bacterial canker is one of the most destructive diseases of apricot in Iran. Copper-based compounds are widely used to protect plants against bacterial diseases, but pathogens frequently evolve resistance against copper (Cu). This study was conducted to investigate the genetic diversity of the Cu resistance gene *cop*A among strains of the causal agent of canker disease of stone fruits *Pseudomonas syringae* pv. *syringae* (Pss), isolated from apricot trees in East Azarbaijan province of Iran. The phylogenic trees based on *cop*A and *rpo*D are very similar to each other revealing no evidence of recombination. To test the hypothesis that pathogenicity- and virulence-related genes may be induced by Cu in Pss strains, quantitative real-time polymerase chain reaction (qRT-PCR) was used to evaluate the expression of genes *alg*D, *cop*A, *fli*C, *hrp*A, *syr*B and *tat*C, in the Cu sensitive Pss 170 strain upon exposure to Cu. Among the tested genes, the *alg*D and *cop*A involved in alginate synthesis and Cu resistance, respectively, showed the highest increase in expression compared to a non-copper (control): 4.75-fold and 2.68-fold, respectively. Based on these results and on the conservation of genes *alg*D and *cop*A in *Pseudomonas* pathovars, antimicrobials that target AlgD and CopA proteins should be developed to use in combination with Cu to increase control efficiency.

Key words: *alg*D gene, *cop*A gene, gene expression, real-time PCR, stone fruit canker.

Introduction

Pseudomonas syringae strains have been isolated from over 180 plant species (Kennelly et al., 2007). *P. syringae* strains are assigned to more than 50 different pathovars based on host range and 13 phylogroups based on phylogeny (Berge et al., 2014). *P. syringae* pv. *syringae* (Pss) van Hall is one of the most destructive causal agents of canker diseases on stone fruit trees. The disease causes yield reductions of up to 80% (Kennelly et al., 2007). In Iran, the disease is currently found in most stone fruit orchards in the country (Najafi Pour Haghighi, Taghavi, 2014).

Copper-based compounds have been widely used for management of bacterial diseases caused by *Pseudomonas* spp., including canker disease of stone fruits (Wimalajeewa et al., 1991). Copper homeostasis systems act as Cu resistance mechanisms and are based on intracellular and extracellular sequestration, enzymatic detoxification, reduced Cu transportation, enhanced efflux of cupric ions, or Cu complexation by cell components (Rademacher, Masepohl, 2012). Essential Cu metabolism-related genes that aid in Cu detoxification are usually located on the main chromosome (Hwang et al., 2005). Copper resistance genes instead can be located either on the main chromosome or on plasmids. Plasmid-determined Cu resistance has been found in diverse Gram-negative bacterial species, such as *P. syringae*, *Xanthomonas campestris* and *Escherichia coli*. In *P. syringae*, plasmid-encoded Cu resistance genes have been identified in various pathovars (Cazorla et al., 2002).

P. syringae pv. *tomato* DC3000 was the first intensively-studied Cu resistant plant pathogenic bacterial strain (Cervantes, Gutierrez-Corona, 1994). In this strain, Cu resistance is encoded by the plasmid pPT23D, which is highly conserved among *P. syringae* strains including strains of Pss (Scheck et al., 1996). This plasmid contains a Cu-resistance operon (*cop*ABCDRS) regulated by a Cu-inducible promoter. Four genes (*cop*A, *cop*B, *cop*C and *cop*D) encode structural components and two genes (*cop*R and *cop*S) have regulatory functions (Mills et al., 1993). *CopA* and *CopB* act as efflux ATPase, while *CopC*

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and *CopD* are responsible for Cu uptake (Rademacher, Masepohl, 2012).

Exposure to Cu may affect expression of pathogenicity and virulence genes that encode toxins, such as syringomycin (Scholz-Schroeder et al., 2001). Other virulence genes encode effector proteins that are translocated into plants by the type III secretion system (T3SS), which is encoded by *hrp* genes (Hwang et al., 2005; Ichinose et al., 2013). The *hrp* genes include *hrpA*, which encodes HrpA, the building block of the translocation pilus (Preston et al., 1995). *P. syringae* strains also produce two exopolysaccharides: alginate and levan (Laue et al., 2006). The *alg*D is the first gene in the alginate biosynthetic gene cluster in *P. syringae* (Peñaloza-Vázquez et al., 1997). Copper has been found to trigger alginate gene expression in Pss (Kidambi et al., 1995). In fact, alginate can sequestrate Cu ions using electrostatic interactions and keep them trapped outside the cell (González et al., 2010). The twin-arginine translocation (Tat) system also contributes to virulence and pathogenicity (Bronstein et al., 2005). The *tat* operon comprises the *tat*ABC genes. TatC is the most conserved of the Tat proteins and acts as an initial receptor for substrate proteins (Alami et al., 2003). Bronstein et al. (2005) reported that a *tat*C gene mutant of DC3000 strain showed sensitivity to Cu and had attenuated virulence. In strain DC3000, the protein CopA has been identified as substrate of a Tat system, which is transported to the periplasm in a Tat-dependent manner (Bronstein et al., 2005). Finally, the bacterial flagellum mediates adherence, which is another important virulence trait in host colonization and flagellin monomers encoded by the *fliC* gene (Rossez et al., 2015) are important inducers of plant defence responses (Haiko, Westerlund-Wikström, 2013).

The purpose of this study was the investigation of genetic diversity and phylogenetic relationships among *Pseudomonas syringae* pv. *syringae* (Pss) strains using the *rpo*D housekeeping gene as well as the Cu resistance *cop*A gene. Also, the effect of Cu on the expression of a selection of the pathogenicity and virulence-related genes: *alg*D, *cop*A, *fli*C, *hrp*A, *syr*B and *tat*C, was determined in a Cu sensitive Pss strain, which had been identified as a causal agent of canker disease of apricot.

Materials and methods

Isolation and characterization of bacterial strains. Bacterial strains were isolated from apricot trees in East Azarbaijan province, Iran, in March and April 2015. Tissues that appeared infected, including buds, blossoms, twigs and branches, were selected for isolation. To isolate bacterial strains epiphytically and endophytically, 5 g of crushed tissues were suspended in 20 ml of 0.1 M potassium phosphate buffer and 0.01 M Mg buffer for 10 and 120 min, respectively, on a shaker at 150 rpm. 100 µl of suspension was streaked on nutrient agar (NA) medium (Merck, Germany) and King's medium B agar (Biolife, Italy), amended with cyclohexamide (KBC) and incubated at 25°C for three days. Then, morphologically different bacterial colonies were classified using Gram staining and fluorescent production on KB (King's B) medium. For identification of *P. syringae* pv. *syringae* (Pss) strains, purified Gram-negative fluorescent colonies were analysed using levan production, oxidase reaction, pectolytic activity on potato slices, arginine dihydrolase activity and hypersensitivity reaction on tobacco leaves (LOPAT test) as well as gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and tartrate utilization (GATTa test) (Schaad et al., 2001). Syringomycin production and ice nucleation activity were tested according to Schaad et al. (2001).

Pathogenicity test. Twenty-four hours old culture of bacterial strains with a concentration of 10^7 colony forming unit (CFU) ml^{-1} and sterile double distilled water (DDW) were used in pathogenicity test as positive and negative controls, respectively, on one-year-old apricot twigs. Twigs in leaf germination sites were inoculated with 1 ml of bacterial suspension and then maintained in high moisture conditions at 28°C for 14 days. The presence of necrotic lesions was considered an indication of pathogenicity (Mohammadi et al., 2001).

Copper (Cu) resistance and growth curves. Mannitol-glutamic yeast (MGY) extract agar is a standard medium used to evaluate Cu resistance *in vitro*. Overnight cultures of each strain on NA medium were suspended in DDW (optical density (OD600) = 0.5). Ten µl of bacterial suspensions in duplicate were spotted on MGY agar supplemented with filter-sterilized (0.45 µm) stock solutions of Cu(II) sulphate pentahydrate (Merck Millipore, Germany) at 19 concentrations: 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.75, 1.0, 1.5, 2, 2.5, 3.0, 3.5 and 4.0 mM. The plates were incubated at 28°C for 72 h. Each assay was performed in triplicate. The minimum inhibitory concentration of copper (MIC-Cu) was defined as the lowest concentration of Cu, at which no growth was observed following incubation at 28°C. Strains with MICs less than 0.75 mM $CuSO_4$ were scored as Cu sensitive (Cazorla et al., 2002).

Growth of *Pseudomonas syringae* pv. *syringae* 170 strain was monitored under Cu conditions using MGY broth medium. The experiments were performed in 250 ml Erlenmeyer flasks containing 70 ml MGY broth medium with three concentrations of copper sulphate ($CuSO_4$): 0, 0.50 and 0.75 mM. Ten µl of bacterial grown (OD600) = 0.6) on nutrient broth (NB) medium (Merck, Germany) were added to the flasks. The flasks were incubated at 28°C with shaking at 150 rpm. Bacterial growth was monitored by measuring the optical densities of bacterial cultures using a spectrophotometer (Eppendorf, Germany) at 600 nm sampling every hour for 196 h.

Detection of genes in Pss strains. CTAB (cetyltrimethylammonium bromide) method according to Doyle and Doyle (1990) with minor modifications was used for total DNA extraction and purification of bacterial strains. A NanoDrop Nano-200 (Allsheng, China) and gel electrophoresis were used for determining of concentration and quality of DNA. The primer pair rpoD-Fp/rpoD-Rp was used to amplify a fragment of the housekeeping gene *rpo*D (Sarkar, Guttman, 2004). The primer pair B1/B2 (Sorensen et al., 1998) was used to amplify a fragment of the syringomycin synthesis gene *syrB*. The custom-designed primers PsscopAF/PsscopAR and PsscopBF/PsscopBR were provided by Macrogen Inc. (South Korea) and were used to amplify fragments of internal regions of the Cu resistance genes *cop*A and *cop*B, respectively. Properties of primers and DNA amplification conditions are summarized in Table 1. All polymerase chain reaction (PCR) assays were performed using a thermocycler Nano-200 (Peqlab Biotechnologie GmbH, Germany) in a final volume of 25 µL containing 12.5 µL of 2× Master Mix Red (Ampliqon, Denmark), 10 pmol of each primer for the respective gene, 8.4 µL H_2O and 2 µL of template DNA.

Sequence alignment and phylogenic analysis. PCR products of the *rpo*D and *cop*A genes were sequenced in both directions (Macrogen Inc.). The program *FinchTV*, version 1.4.0 (Geospiza Inc., USA) was used to check the quality of raw sequencing reads. Then, software *SeqMan™ II* (DNASTAR Inc., USA) was used for editing and trimming of raw sequencing data. Obtained sequences were used to search the NCBI database (www://ncbi.nlm.nih.gov) using the BLASTN (megablast) tool. The MUSCLE algorithm implemented in the software *MEGA*, version 6 (Tamura et al., 2013) was used for sequence aligning. Sequences were aligned using the MUSCLE algorithm implemented in the software *MEGA 6* (Tamura et al., 2013).

Phylogenetic trees were constructed using software *Mesquite*, version 2.75 (Maddison, Maddison, 2011). Phylogenic analysis was performed using program

Table 1. Gene fragments, primers and polymerase chain reaction (PCR) conditions used in this study

Target gene	Primer name	Primer sequence 5′ to 3′	PCR conditions	Amplicon size (bp)	Reference
*rpo*D	rpoD-Fp rpoD-Rp	AAGGCGARATCGAAATCGCCAAGCG GGAACWKGCGCAGGAAGTCGGCACG	95°C 5 min; 30 cycles (94°C 1.5 min, 63°C 1 min and 72°C 2 min); 72°C 10 min.	532	Sarkar, Guttman, 2004
*syr*B	B1 B2	CTTTCCGTGGTCTTGATGAGG TCGATTTTGCCGTGATGAGTC	94°C 4 min; 35 cycles (94°C 1.5 min, 60°C 1.5 min and 72°C 3 min); 72°C 10 min.	752	Sorensen et al., 1998
*cop*A	PsscopAF PsscopAR	CCTGCCATACCCATATCATCC CTAGTGATCGACGCGAAAGAG	95°C 5 min; 35 cycles (94°C 90 s, 55°C 60 s and 72°C 2 min); 72°C 10 min.	650	This study
*cop*B	PsscopBF PsscopBR	CTGGGTGTCTTCGTCGTCTT GCTGGATTGGCGGAGATA	95°C 5 min; 35 cycles (94°C 90 s, 55°C 60 s and 72°C 2 min); 72°C 10 min.	506	This study

MrBayes, version 3.2.2 using a Markov Chain Monte Carlo (MCMC) algorithm (Ronquist et al., 2012) with 100,000,000 generations, average standard deviation of split frequencies value of 0.01, sampling every 1000 generations, and setting of the heating parameter to 0.15. After discarding the first 25% of the generations as "burn-in", the posterior probabilities were calculated from the remaining trees. The Akaike information criterion (AIC) implemented in program *MrModeltest*, version 2.3 (Nylander, 2004) was used to select the best model of nucleotide substitution independently for each locus. The program *FigTree*, version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) was used to visualize the trees.

RNA extraction. A 90 µl inoculum from a culture during exponential growth of the Pss 170 strain in NB medium (Merck, Germany) was added to 70 ml MGY broth medium without Cu supplementation in a sterile 250 ml Erlenmeyer flask. Bacterial cells were grown in MGY broth medium at 28°C and 150 rpm until they reached an optical density at 600 nm of 0.6 during exponential growth with shaking at 150 rpm. At this point, two concentrations of $CuSO_4$, H_2O, 0.50 and 0.75 mM, were added to the cultures. Control cultures without the addition of Cu were also obtained. Bacterial cultures were maintained at 28°C and 150 rpm. Five ml samples were centrifuged at 5000× g at 4°C for 15 min at 2, 4, 8 and 12 h after addition of Cu. Total RNA was extracted immediately using RNX-Plus Solution (SinaClon Co., Iran) as described by the manufacturer. RNA extractions were performed in triplicate. All RNA samples were analysed for quantity using the NanoDrop Nano-200 (Allsheng) and the integrity of the RNA was determined by visualization after gel electrophoresis. Genomic DNA was removed by treatment of RNA samples with RNase-free DNase I (Thermo Fisher Scientific, USA) for 30 min at 37°C.

Complementary DNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR). Complementary DNA synthesis was performed as described by the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Samples of cDNA obtained from three independent replicates of each treatment were pooled. The pooled cDNA served as a single biological replicate for each treatment condition and used in triplicate qRT-PCR. It was performed using the RealQPCR 2× SYBR Green Master Mix (Ampliqon) in a StepOne™ Real-Time PCR System (Applied Biosystems®). Gene-specific primers for the genes *alg*D, *cop*A, *fli*C, *hrp*A, *rpo*D, *syr*B and *tat*C were designated using the program *AlleleID*, version 6.0 (Premier BioSoft, USA). The qRT-PCR primers were designed in order to have a length of about 20 to 25 bases, a G/C content of over 50%, Tm of 60°C and the length of the PCR product ranged from 90 to 130 bp. The *rpo*D gene was used as an internal reference as in previous qRT-PCR experiments (Hockett et al., 2013).

Primer sequences are shown in Table 2. For each of the 12 independent pooled cDNA samples, gene expression was measured in three technical replications and non-template control. The mean of these values was used for further analysis.

The PCR reaction mixtures in a total volume of 15 µl contained 7.5 µl of SYBR Green RT PCR Master Mix, 10 pmol of forward and reverse primers and 5 µl of cDNA template. The amplification conditions were as follows: one cycle of initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, at 56°C for 25 s and at 72°C for 30 s. The specificity of all primer pairs and all amplification reactions was confirmed by single-peak melting curve analysis (a denaturing step at 95°C for 15 s, a hybridization step of 1 min at 70°C, followed by temperature increases of 0.3°C per cycle from 70°C to 95°C with a 5 s stop between each step). Gel electrophoresis and melting curve analysis of PCR products showed that very little or no primer dimers were generated. All raw values were normalized to the *rpo*D gene, which was used as internal reference gene. The relative expression ratios were calculated following the model of Pfaffl (2001), which included an efficiency correction for real-time PCR efficiency of the individual transcripts:

$$\text{Ratio} = (E_{\text{target gene}})^{\Delta Ct}{}_{\text{target(control}-\text{sample)}} / (E_{\text{reference gene}})^{\Delta Ct}{}_{\text{ref(control}-\text{sample)}},$$

where E is the real-time PCR efficiency for a given gene, Ct – the crossing point of the amplification curve with the threshold, ΔCt – the crossing point

Table 2. Primers for quantitative real-time polymerase chain reaction (qRT-PCR) used in this study

Gene	Function	Primer name	Primer sequence 5' to 3'	Amplicon size	Efficiency
*alg*D	Alginate synthesis	qRTalgDF qRTalgDR	GATAGTGTGGCGGGTGGCTTT AAGAACGGCGATCTGGAACTGG	90 bp	90%
*cop*A	Copper resistance	qRTcopAF qRTcopAR	GTCATTGCCGAGCCGTTG GCGACCTACACCTACCTGATG	110 bp	90%
*fli*C	Flagellin	qRTfliCF qRTfliCR	ACTCGCAGATCAAAGGTCAG AGCCAGTTCACGCATACG	125 bp	92.5%
*hrp*A	Hrp pilus	qRThrpAF qRThrpAR	CGCCGACCGTAATGCTG GCCGTTCTCTTCGTTCGC	130 bp	90%
*rpo*D	Sigma factor 70	qRTrpoDF qRTrpoDR	GCCAGCGACGAAGAAGAC GCCTTGCGGGTGATTTCC	101 bp	92.5%
*syr*B	Syringomycin synthesis	qRTsyrBF qRTsyrBR	ACGCTCGGGCTGGTTTGC CGACCTGATGACCCTGGAGAG	129 bp	90%
*tat*C	Twin-arginin translocase	qRTtatCF qRTtatCR	CGATGAATGACGACGACAAG ATGGTGTGCGGCTGATTC	100 bp	90%

difference for an unknown sample *versus* a control. For each individual gene, the real-time PCR efficiencies were assessed using software *LinRegPCR* (Rutledge, Stewart, 2008). Amplification efficiencies were determined to be 90% and 92.5%, and were consistent between gene targets.

Expression levels of genes are presented as the mean of three replicates. For statistical analysis, data were analysed for variance using ANOVA, and the means were compared by Duncan's multiple range test using statistical software *MSTAT-C*, version 1.42 (Michigan State University, USA). Level of significance for different treatments was determined at 5% probability ($P < 0.05$).

Results

Bacterial isolation and identification. A total of 103 Gram-negative strains were isolated. Five fluorescent and aerobic strains were identified as Pss based on the LOPAT (levan production, oxidase reaction, pectolytic activity on potato slices, arginine dihydrolase activity and hypersensitivity reaction on tobacco leaves) test (+, −, −, −, +) and GATTa (gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and tartrate utilization) test (+, +, −, −). The five strains had been collected from different geographic areas of Iran, including Ajabshir (Pss 26), Marand (Pss 82) and Sepidan (Pss 170, Pss 174 and Pss 176). With the exception of Pss 176, other Pss strains were isolated epiphytically. All five Pss strains produced syringomycin like toxins based on their ability to inhibit mycelial growth of *Geotrichum candidum* were ice nucleation active and were pathogenic on one-year-old apricot twigs inducing severe necrotic lesions at injection sites (Table 3). Large bacterial populations showing the same colony morphologies were re-isolated from infected tissues and confirmed to be Pss based on biochemical tests and PCR.

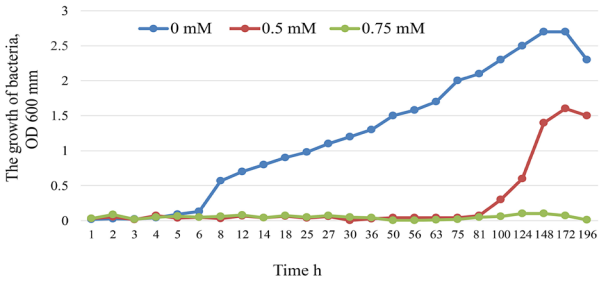
Table 3. *Pseudomonas syringae* pv. *syringae* (Pss) strains isolated from infected apricot tissues used in this study

Strains	Geographical areas	Kind of isolation	LOPAT results					GATTa results				Pathogenicity test	Ice nucleation activity	Syringomycin production	MIC-Cu	Detection of *syr*B
			L	O	P	A	T	G	A	T	T					
Pss 26	Ajabshir	Epiphytic	+	−	−	−	+	+	+	−	−	+	+	+	0.50 mM	+
Pss 82	Marand	Epiphytic	+	−	−	−	+	+	+	−	−	+	+	+	0.75 mM	+
Pss 170	Sepidan	Epiphytic	+	−	−	−	+	+	+	−	−	+	+	+	0.75 mM	+
Pss 174	Sepidan	Epiphytic	+	−	−	−	+	+	+	−	−	+	+	+	0.50 mM	+
Pss 176	Sepidan	Endophytic	+	−	−	−	+	+	+	−	−	+	+	+	0.75 mM	+

MIC-Cu – minimum inhibitory concentration of copper

Copper (Cu) resistance of Pseudomonas syringae pv. syringae (Pss) strains on solid medium. To assess the antimicrobial activity of copper, MIC-Cu was evaluated. The results showed that the lower concentrations (<0.50 mM) of Cu did not cause any appreciable effect on bacterial growth. However, two strains (Pss 26 and Pss 174) were inhibited by 0.50 mM of Cu and three strains (Pss 82, Pss 170 and Pss 176) were inhibited by 0.75 mM of Cu. No growth was observed for any Pss strains at 1 to 4 mM of Cu.

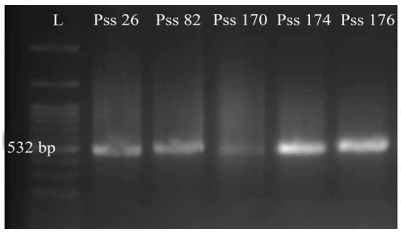
Growth of Pss 170 at different concentrations of Cu. The growth curve of the Cu sensitive Pss 170 strain was determined at two different concentrations (0.50 and 0.75 mM) of Cu in MGY broth medium compared to a control (without Cu). In the absence of Cu, Pss 170 reached the exponential phase (OD600 = 0.6) after 10 h, in the 0.50 mM of Cu, Pss 170 reached the exponential phase (OD600 = 0.6) after 124 h, but in the 0.75 mM of $CuSO_4$ the bacteria stayed in lag phase during the entire 196 h of the experiment (Fig. 1).



OD – optical density

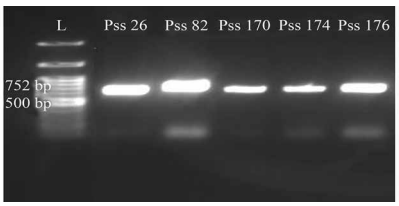
Figure 1. Growth curve of *Pseudomonas syringae* pv. *syringae* 170 strain (Pss 170) in presence of two concentrations (0.50 and 0.75 mM) of Cu compared with control (absence of Cu) in MGY broth medium

Detection of rpoD, syrB, copA and copB genes in Pss strains. Using rpoD-Fp/rpoD-Rp primers, a 532 bp fragment was amplified from all collected Pss strains (Fig. 2). The presence of the *syr*B gene using B1/B2 primers was verified by amplification of a 752 bp fragment in all strains (Fig. 3). The presence of the Cu resistant genes *cop*A and *cop*B was verified by amplification of 650 bp (Fig. 4) and 506 bp fragments in all Pss strains (Fig. 5) using the designed PsscopAF/R and PsscopBF/R primers, respectively.



L – 100 bp DNA ladder

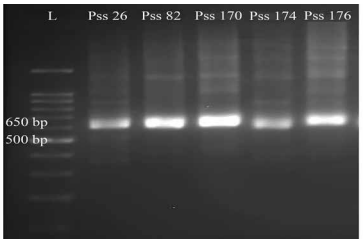
Figure 2. Agarose 1% gel electrophoresis of PCR products of *Pseudomonas syringae* pv. *syringae* (Pss) strains using rpoD-Fp/rpoD-Rp primers



L – 100 bp DNA ladder

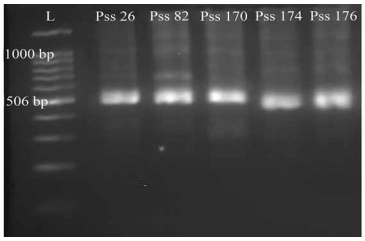
Figure 3. Agarose 1% gel electrophoresis of PCR-amplified gene coding syringomycin in *Pseudomonas syringae* pv. *syringae* (Pss) strains using B1/B2 primers

Sequence alignment and phylogenetic analysis. Partial nucleotide sequences of the *rpo*D and *cop*A genes obtained from different Cu sensitive strains of Pss were used for phylogenetic analysis. The average sequence length obtained from amplification was 545 and 620 bp for the *rpo*D and *cop*A gene, respectively. Edited *rpo*D and *cop*A gene sequences were aligned to the sequences deposited in NCBI's GenBank database (https://www.ncbi.nlm.nih.gov/). The alignment of the partial nucleotide sequences of the Cu resistance gene revealed homology of over 96% for the *cop*A gene among all reference Pss strains in NCBI's GenBank.



L – 100 bp DNA ladder

Figure 4. Agarose 1% gel electrophoresis of PCR-amplified gene coding syringomycin in *Pseudomonas syringae* pv. *syringae* (Pss) strains using PsscopAF/ PsscopAR primers



L – 100 bp DNA ladder

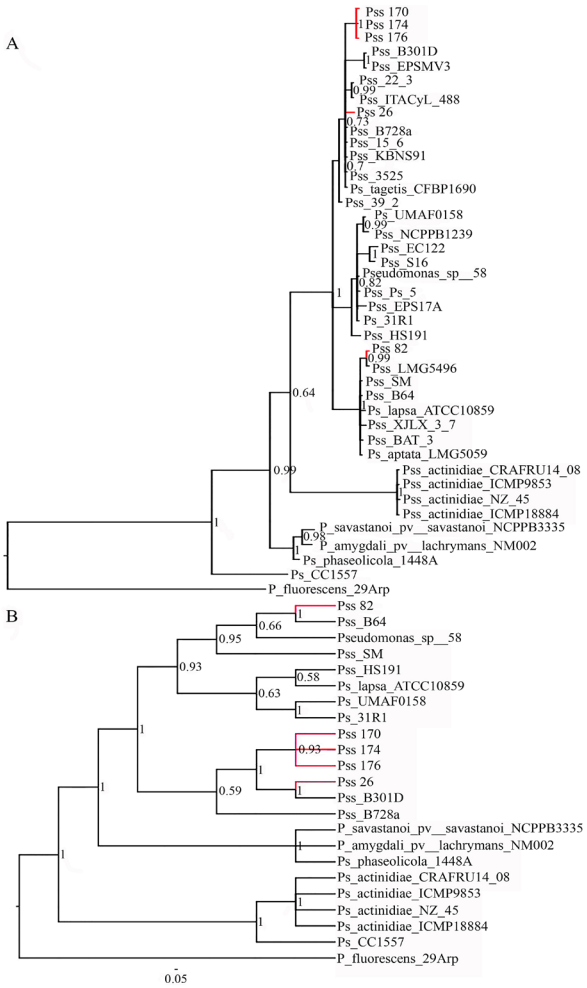
Figure 5. Agarose 1% gel electrophoresis of PCR-amplified gene coding syringomycin in *Pseudomonas syringae* pv. *syringae* (Pss) strains using PsscopBF/ PsscopBR primers

The best-fit statistical model was determined for each locus based on results from program *MrModeltest 2.3*. General time reversible with gamma distribution (GTR + G) model was recommended for both gene fragments. Phylogenetic trees using Bayesian inference method were constructed to evaluate the evolutionary relationships of the five isolated Pss strains with reference strains based on individual sequences of the *rpo*D (532 nucleotides) and *cop*A (599 nucleotides) genes.

The topology of the *cop*A gene phylogeny was in agreement with the *rpo*D gene phylogeny. In the phylogenetic trees based on the *rpo*D and *cop*A genes, all Iranian Pss strains clustered into two main groups (Fig. 6). Iranian strains in the main group I were divided into two subgroups. Subgroup I included the Pss 170, Pss 174 and Pss 176 strains collected in Sepidan, and subgroup II included the Pss 26 strain collected in Ajabshir. Group II included the Pss 82 strain collected in Marand area. The *Pseudomonas fluorescens* R124 strain was used as outgroup. These biomarkers separated strains collected from the apricot orchards in different geographic areas into different groups and subgroups.

Expression of the selected pathogenicity and virulence-related genes. Because little is known about the regulation of the Cu stress response in Pss strains, the Cu sensitive Pss 170 strain was exposed to elevated concentrations of Cu to examine the responses of pathogenicity and virulence-related genes for up to 12 h using qRT-PCR. The level of relative gene expression was estimated based on the cycle threshold (Ct) values using standard curves. In all cases, the data were normalized relative to the *rpo*D gene. This housekeeping gene was chosen as an internal control, because previous experiments had shown that its transcript levels were not significantly altered under different conditions. Results are shown in Figure 7.

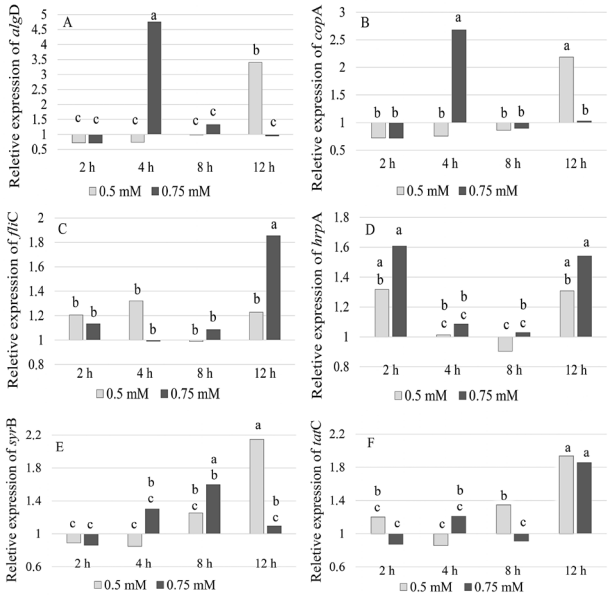
The *alg*D gene showed a transcript level range of 0.71 to 4.75 in relative expression. Analysis of results showed that there was a statistically significant difference among treatments at the 5% level (Fig. 3A). In 0.75 and 0.50 mM Cu, *alg*D gene expression showed up-regulation of 4.75-fold and 3.4-fold compared to the control (without Cu) after 4 and 12 h, respectively. Analysis of



Note. The scale bar represents the average number of substitutions per site, and posterior probability values are shown at the nodes obtained for 100000000 replicates; Iranian strains were shown using pink branches; Pss – *Pseudomonas syringae* pv. *syringae*, Ps – *Pseudomonas syringae*, Pf – *Pseudomonas fluorescens*.

Figure 6. Phylogenetic trees constructed by Bayesian inference method based on partial sequences of housekeeping RNA polymerase *rpo*D (A) and Cu resistance *cop*A (B) genes among Ps strains using GTR + G model

relative expression results of the *cop*A gene showed that there was statistical difference among treatments at the 5% level (Fig. 3B). The *cop*A gene showed up-regulation in 0.75 and 0.50 mM of Cu after 4 and 12 h up to 2.68-fold and 2.18-fold compared to the control, respectively. The *fli*C gene showed up-regulation most of the time at both Cu concentrations compared to control. Statistical analysis of the ratio obtained from relative expression of the *fli*C gene showed that there was difference among treatments at the 5% level (Fig. 3C). The highest increase was 1.85-fold after 12 h of exposure to 0.75 mM Cu compared to the control. For the *hrp*A gene, statistical differences were detected based on the analysis of relative expression among treatments at the 5% level (Fig. 3D). At both Cu concentrations, the highest up regulation (1.3-fold and 1.6-fold in 0.50 and 0.75 mM, respectively) were detected at 2 and 12 h compared to the control. The *syr*B gene showed up-regulation 2.14-fold and 1.6-fold compared to the control in 0.50 and 0.75 mM after 12 and 8 h, respectively, whereby differences were statistically significant among treatments at the 5% level (Fig. 3E). Analysis of ratios in relative expression of the *tat*C gene showed that there was a statistically significant difference among treatments at the 5% level (Fig. 3F).



Note. Fold induction relative to the control condition (0 mM $CuSO_4$) is expressed in 2, 4, 8 and 12 h after addition of the Cu; the relative expression values indicated in the graph are the average of three replicates; ANOVA was calculated using software *MSTAT-C* and Duncan's multiple range test; $P < 0.05$ was considered statistically significant among means.

Figure 7. The relative expression levels of genes *alg*D (A), *cop*A (B), *fli*C (C), *hrp*A (D), *syr*B (E) and *tat*C (F) were quantified in Cu sensitive *P. syringae* pv. *syringae* 170 strain (Pss 170) cells in presence of 0.50 and 0.75 mM of $CuSO_4$ using qRT-PCR method

The highest up regulation of the *tat*C gene was 1.93-fold and 1.85-fold, which were obtained after 12 h of Cu exposure compared to control in 0.50 and 0.75 mM Cu, respectively.

Discussion

Pseudomonas syringae pv. *syringae* (Pss), the causal agent of bacterial canker of stone fruit, colonizes trees both epiphytically and endophytically. The epiphytic and endophytic phases play important roles in disease epidemiology: the epiphytic population size of the pathogen is associated with disease incidence and severity, because the epiphytic population of Pss on surfaces of apparently healthy blossoms and leaves provides the inoculum for the endophytic phase of the disease. Four of the five Pss strains collected in this study were isolated epiphytically.

Copper-based antimicrobial compounds are commonly used for controlling bacterial diseases, but there are limitations to their usage: emergence of resistant strains, lack of systemic activity and phytotoxicity. Copper treatments that target epiphytic populations can reduce the size of the epiphytic population and limit the potential increase of the inoculum, thereby reducing disease severity (Wimalajeewa et al., 1991). All Pss strains collected in this study showed ice nucleation activity. The epiphytic phase and the ice nucleation activity followed by injury are both known predisposing factors for canker initiation and development in stone fruit trees (Renick et al., 2008).

All five strains identified here were found to be sensitive to $CuSO_4$ with minimum inhibitory concentrations (MICs) from 0.50 to 0.75 mM. The different reactions of Pss strains to MIC-Cu were apparently not related to the geographical origins. In fact, strains isolated from the same geographical area, such as Sepidan, showed different MICs to Cu. The growth rate of Pss 170 strain showed that when the Cu concentration was increased from 0.50 to 0.75 mM (as MIC), the exponential phase (OD600 = 0.6) was significantly delayed. The MIC determined on solid media may not be the same as the one in liquid media, because some characters, such as agar and pH, can affect metal solubility and availability, respectively (Hartley et al., 1997).

A phylogenetic tree was built based on partial sequences of the Cu resistance gene *cop*A of the Iranian Pss strains and *P. syringae* reference strains with alignment homology of over 86% (E value = 0.0). In this tree, Iranian strains were placed in two separated groups. Placement of the Iranian strains within the tree correlates with geographic origin so that strains (Pss 170, Pss 174 and Pss 176) isolated from Sepidan cluster in a single subclade with high posterior probability and are separated from strains isolated from Ajabshir (Pss 26) and Marand (Pss 82) areas. The Cu sensitive Pss 82 strain in group I clustered with high posterior probability with the Cu resistant Pss B64 reference strain. The strain was isolated from wheat and is ice nucleation active (Hwang et al., 2005). Iranian strains in group II (Pss 170, Pss 174, Pss 176 and Pss 26) are most closely related to two reference strains, Pss B301D and Pss B728a, which were isolated from pear and pea, respectively. Iranian strains in group II were Cu sensitive (MIC < 0.8 mM Cu), ice nucleation active and able to produce syringomycin. Based on previous studies, the two reference strains in this group are Cu resistant (MIC > 0.8 mM Cu) and are also able to produce syringomycin and are ice nucleation active (Ravindran et al., 2015). Li et al. (2015) hypothesized that the polymorphic Cu resistance gene *cop*A can be divided into two highly conserved groups encoding for multicopper oxidase and P-type ATPase.

In this study, *P. syringae* strains in the phylogenetic tree based on the alignment of the protein CopA were placed in both, the P-type ATPase and the multicopper oxidase groups. In our study, all Iranian Pss strains clustered with the *P. syringae* refrence strains Pss 64, Pss B728a and Pss 301D encoding for P-type ATPase. CopA is part of the Cu-exporting P-type ATPase IB group of heavy metal transport ATPases (Petersen, Moller, 2000).

The topography of the tree based on partial sequences of the housekeeping gene *rpo*D was similar to the Cu resistance *cop*A gene. In the phylogenic tree based on *rpo*D, Iranian strains were grouped the same way as in the *cop*A tree. Hwang et al. (2005) reported that the ability of detoxification of Cu appears to be an ancestral trait in *P. syringae* strains. In our study, the presence of *cop*A gene in all studied Pss strains agreed with detoxification of Cu being an ancestral trait in *P. syringae*.

Extracellular polysaccharide (EPS) production, such as that of alginate, has been associated with virulence of Pss strains due to its role in increased epiphytic fitness, facilitation of colonization and/or dissemination *in planta*, induction of water-soaked lesions on infected leaves, adhesion to plant surfaces, biofilm formation and resistance to toxic molecules and dehydration (Yu et al., 1999; O'Brien et al., 2011). Alginate biosynthesis and secretion genes are conserved in the genomes of some Pss strains (Ravindran et al., 2015). In the present study, the highest expression of *alg*D gene was found at 4 and 12 h post-exposure to Cu in 0.75 and 0.50 mM Cu, respectively. Our results are consistent with Kidambi et al. (1995) who reported that Cu-based compounds applied for control of bacterial diseases in plants trigger alginate gene expression and thus increase alginate production in a number of Pss strains upon exposure to Cu ions.

In previous research, it had been demonstrated that at high concentrations of Cu, defences based on the P-type Cu export ATPase are critical virulence factors in pathogenic bacteria (Argüello et al., 2011). In our study, the expression level of *cop*A showed an over 2-fold up-regulation compared to control at 12 h in 0.50 mM of Cu and at 4 h in 0.75 mM Cu. A similar up-regulation of

the Cu resistance genes *cop*A and *cop*B was reported in *Xanthomonas axonopodis* pv. *citri* (Palmieri et al., 2010). These results can be explained by the role of these genes in bacterial resistance to antimicrobial compounds, which requires a rapid response to increasing Cu concentrations. The present study is the first to show enhanced expression of virulence-related *hrp*A, *fli*C and *syr*B genes under Cu stress in any *P. syringae* strain. This is similar to results obtained by Palmieri et al. (2010) for *X. axonopodis* pv. *citri*, in which virulence-related (*xcs*H and *xcs*C) genes showed enhanced expression in the presence of Cu.

In different bacterial pathogens, participation of the twin-arginine translocation (TAT) system has been shown in both, assembly and function of the flagellum as well as in secretion of cofactor-bound proteins and much virulence factors (Ochsner et al., 2002). In this study, *tat*C gene expression was up-regulated following inoculation with Cu. Up-regulation of the *tat*C gene may improve secretion of the Cu resistance protein CopA since previous studies identified the protein CopA as a substrate of the twin-arginine translocation system in strain DC3000 (Bronstein et al., 2005). Also, in the protein PcoA of *P. aeruginosa*, a CopA orthologue, a TAT signal sequence with twin arginine residues was identified (Vasil et al., 2012). Moreover, Ochsner et al. (2002) showed that Cu increased inhibition of growth in a *P. aeruginosa tat*C gene mutant compared to wild-type and the observed increased susceptibility to Cu was attributed to the protein PcoA.

Based on results of our experiment, the high expression of the genes *alg*D and *cop*A under Cu stress, their sequence conservation among *Pseudomonas* strains (Muhammadi, Ahmed, 2007) and essential role in defence against Cu suggest that novel antimicrobial compounds could be developed to interfere with the function of proteins AlgD and CopA to increase susceptibility of Pss to Cu.

Conclusions

1. All collected *Pseudomonas syringae* pv. *syringae* (Pss) strains were copper (Cu) sensitive with minimum inhibitory concentration (MIC) less than 0.75 mM.

1. In the phylogenic tree based on the *cop*A gene, all Iranian Pss strains clustered with the Pss reference strains encoding for CopA protein as a part of the Cu-exporting P-type ATPase.

2. Based on our knowledge, the present study is the first to evaluate the expression of *fli*C, *hrp*A and *syr*B genes under Cu stress in any Pss strain.

3. Based on gene expression results, we suggest antimicrobials that target AlgD and CopA proteins could be developed to be used in combination with Cu.

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Vario streso įtaka *Pseudomonas syringae* pv. *syringae* patogeniškumo ir virulentiškumo genų raiškai

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Santrauka

Kaulavaisių bakterinis vėžys yra viena žalingiausių abrikosų ligų Irane. Siekiant augalus apsaugoti nuo bakterinių ligų, plačiai naudojami vario junginiai, tačiau patogenai dažnai įgauna atsparumą variui. Tyrimo tikslas – ištirti kaulavaisių vėžio sukėlėjo *Pseudomonas syringae* pv. *syringae* (Pss) kamienų, išskirtų iš abrikosų medžių Irano Rytų Azerbaidžano provincijoje, genetinę atsparumo variui geno *cop*A įvairovę. Filogeniniai medžiai pagal *cop*A ir *rpo*D genus yra labai panašūs vienas į kitą, o tai rodo, kad tarp jų nėra rekombinacijos. Siekiat patikrinti hipotezę, kad Pss kamienuose varis gali indukuoti genus, susijusius su patogeniškumu ir virulentiškumu, taikyta kiekybinė realaus laiko polimerazės grandininė reakcija (*qRT-PCR*). Siekta įvertinti genų *alg*D, *cop*A, *fli*C, *hrp*A, *syr*B ir *tat*C raišką variui jautriame Pss 170 kamiene veikiant variu. Iš tirtų genų didžiausią raiškos padidėjimą ir atsparumą variui parodė genai *alg*D ir *cop*A, dalyvaujantys alginatų sintezėje – atitinkamai 4,75 ir 2,68 karto, palyginus su kontroliniu variantu be vario. Remiantis tyrimo duomenimis ir *alg*D bei *cop*A genų konservatyvumu *Pseudomonas* patovaruose, siekiant padidinti apsaugos kontrolės efektyvumą, reikėtų sukurti antimikrobines medžiagas, nukreiptas į baltymus AlgD ir CopA, kurios būtų naudojamos kartu su variu.

Reikšminiai žodžiai: *alg*D genas, *cop*A genas, genų ekspresija, kaulavaisių vėžys, realaus laiko PGR.