DETECTION OF GENES RESPONSIBLE FOR HEAVY METALS RESISTANCE IN LOCALLY ISOLATED *PSEUDOMONAS* SPP.

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) that can tolerate heavy metals, provide the basis for microbial inoculums showing heavy metals tolerance properties. This study was aimed to detect the heavy metal resistance genes in plant-growth-promoting *Pseudomonas* spp. isolated from many agricultural fields. The collected isolates were screened for their plant growth-promoting (PGP) traits, hydrolytic enzymes, Siderophore, ammonia, and indole-3-acetic acid (IAA). Then, subjected to concentrations of $CuSO_4$, $CdCl_2$, and $ZnCl_2$ to determine the minimum inhibitory concentration (MIC). The DNA was extracted from the selected isolates then PCR test was achieved to detect *copA*, *copB*, and *czcA* genes, responsible for heavy metal resistance. Seventy *Pseudomonas* spp. isolates were obtained; 41 (58.57%), 6 (8.57%), and 15 (21.42%) isolate produced protease, cellulase, and pectinase, respectively. The isolates were positive for siderophore and ammonia production. However, 68 (97.14%) isolates have produced indole-3-acetic acid. Eight isolates were selected and identified as *Pseudomonas aeruginosa* using the Vitek 2 compact system. The isolates' resistance to heavy metals differed significantly. The isolate B49 had a higher resistance to $CuSO_4$ (MIC = 3200 µg/ml) and $ZnCl_2$ (MIC = 2600 µg/ml), while the isolate B66 recorded a higher resistance to $CdCl_2$ (MIC = 1000 µg/ml). *copB*, and *czcA* genes were detected in the eight *P. aeruginosa* isolates, while *copA* gene was detected in seven, except B69.

Keywords: plant growth promoting rhizobacteria (PGPR), *P. aeruginosa*, *copA*, *copB* and *czcA*.

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الكشف عن الجينات المسؤولة عن مقاومة المعادن الثقيلة في *PSEUDOMONAS* SPP. المعزولة محليًا

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المستخلص

ان بكتيريا الجذور المعززة لنمو النبات (PGPR) التي يمكنها تحمل المعادن الثقيلة،توفر الأساس للقاح ميكروبي يظهر خصائص تحمل المعادن الثقيلة. هدفت هذه الدراسة إلى تقييم صفات تعزيز نمو النبات والكشف عن الجينات المقاومة للمعادن الثقيلة لبكتريا Pseudomonas spp. والتي عزلت من عدة حقول زراعية. تم غربلة العزلات التي تم جمعها لمعرفة خصائصها المعززة لنمو النبات: الإنزيمات الحالة والسايدروفور والأمونيا واندول حامض الخليك. ثم عرضت لتراكيز من $CuSO_4$ و $CdCl_2$ و $ZnCl_2$ لتحديد التركيز المثبط الادنى (MIC). تم استخلاص الحمض النووي (DNA) من العزلات المختارة ثم اجري اختبار تفاعل البلمرة المتسلسل (PCR) للكشف عن جينات مقاومة المعادن الثقيلة ***copA*** و ***copB*** و ***czcA*** . تم الحصول على سبعين عزلة *Pseudomonas* spp. : 41 (58.57٪) و 6 (8.57٪) و 15 (21.42٪) عزلة منتجة للبروتيز والسليليز والبكتينيز بالترتيب ، وجميعها إيجابية لإنتاج السايدروفور والأمونيا. في حين 68 (97.14٪) عزلة انتجت إندول حامض الخليك (IAA). تم اختيار وتشخيص ثمانية عزلات باستعمال نظام الفايتك، وتبين أنها *Pseudomonas aeruginosa*. اختلفت مقاومة العزلات للمعادن الثقيلة معنوياً، وكان للعزلة B49 مقاومة أعلى لكبريتات النحاس (MIC= 3200 مايكروغرام/مل) و كلوريد الزنك (MIC= 2600 مايكروغرام/مل) ، بينما سجلت العزلة B66 مقاومة أعلى لكلوريد الكادميوم (MIC= 1000 مايكروغرام/مل). تم الكشف عن وجود الجينات ***copB*** و ***czcA*** في عزلات *P. aeruginosa* الثمانية، بينما وجد الجين ***copA*** في سبعة عزلات فقط، باستثناء العزلة B69.

الكلمات المفتاحية: بكتريا الجذور المعززة لنمو النبات (PGPR)، *P. aeruginosa* ، *copA* ، *copB* و *czcA*.

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INTRODUCTION

Microorganisms in the rhizospheric soil range from beneficial to pathogens. The beneficial bacteria such as *Pseudomonas* spp. and *Bacillus* spp. have shown a potential role in improving plant growth through nutrient provide and producing many biological control agents to face the disease-causing microorganisms (10, 42). *Pseudomonas* species represent essential PGPR (Plant growth-promoting rhizobacteria) that increase crop yield through direct and indirect methods. PGPR have several processes for controlling plant pathogens and they also compete with phytopathogens for resources and space (14, 38). *Pseudomonas* spp. is ubiquitous bacteria with various applications because they exhibit a many of diverse traits in different environments, was utilized as a biocontrol agent, plant growth promoter, source of antibiotics and efficient bioremediation strains. Plant growth-promoting systems traditionally grouped direct and indirect processes (13). Heavy metals, such as Zn, Cd, Ni, Cu, Pb, Cr and Hg, are a common problem in agricultural soil fields, and it is frequently caused by industrial processes located nearby (9, 20). These metals are hard to remove from the environment, differ from many pollutants that can be decomposed biologically or chemically and are ultimately undegradable, so their harmful effects last longer (3, 6). Bacteria have evolved numerous resistance mechanisms to cope with heavy metal stress. Several resistance mechanisms involve heavy metal, complexation, sequestration, metal conversion to a less hazardous species, and direct metal efflux out of the cell. Metals crossing the bacterial cell may interact in many ways based on their chemical properties and concentration. When exposed to high levels, cells usually respond by expressing unique systems of resistance (P-type ATPases, CDF (cation diffusion facilitator transporters), resistance-nodulation-cell division) RND)efflux pumps, metallothioneins)(35). Heavy metal resistance responsible genes *copA*, *copB*, and *czcA* were found in gram-negative bacteria such as *Pseudomonas syringae, Pseudomonas putida, Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Xanthomonas* spp. CopA is a P-type ATPase found in the periplasmic membrane, and CopB is a copper-binding protein in the outer membrane that protects against copper. The *czcA* gene is a member of the Resistance Nodulation-Division (RND) family found in the periplasm that serves as a metal efflux pump protein for Cd and Zn (16, 23, 29). The objective of the current study was to evaluate *Pseudomonas* spp. isolates as plant growth promoters and heavy metals resistant and detect the presence of genes responsible for their resistance to Cu, Cd, and Zn.

MATERIALS AND METHODS

Samples collection

This study involved four agricultural fields of Karbala, Baghdad, Hilla City, and the College of Agriculture (Iraq), from October 2020 to January 2021. The soils samples were collected from various types of rhizospheric soils of different plants, Banana (*Musa* spp.), Bean (*Phaseolus vulgaris*), Alfalfa (*Medicago sativa*), Wheat (*Triticum aestivum*), Barley (*Hordeum vulgare*), Jerusalem artichoke (*Helianthus tuberosus*), Okra (*Abelmoschus esculentus*), Conocarpus (*Conocarpus* spp.), Mint (*Mentha* spp.), Corn (*Zea mays*), Barley (*Hordeum vulgare*), Wheat (*Triticum aestivum*), Sunflower (*Helianthus annuus*), and Jerusalem artichoke (*Helianthus tuberosus*).

Isolation of *Pseudomonas* spp.

Serial dilutions were prepared from different plants' rhizosphere. 1 g of soil was added to a test tube was contained 9 ml of H_2O (0.85% NaCl), mixed thoroughly, and left to stand before they formed successive serial dilutions. A 100 µl of each 10^{-4}, 10^{-5}, and 10^{-6} dilution was spread on the surface of three agar media: King's B, *Pseudomonas* isolation agar (PIA), and Cetrimide agar, then incubated at 28°C for 48 h and re-cultured on the same media. Different colonies were purified in MacConkey agar medium and subjected to biochemical tests.

Extracellular enzymes production

Protease production was tested using skimmed milk agar, 28 g skimmed milk powder, 1 g Dextrose, 5 g Tryptone, 2.5 g Yeast extract, and 15 g Agar (g L-1). The isolates that formed clear zones, positive results, around the colony were recorded after 24h of incubation at 28°C (15). The test of cellulase production was done using minimal medium, 2 g tryptone,

4 g Na_2HPO_4, 4 g KH_2SO_4, 10 g CMC, 0.2 g $MgCl_2$, 0.004 g $FeSO_4$, 0.001 g $CaCl_2$, 20 g agar (g L^{-1}), combined with 1% carboxymethyl cellulose (CMC). After six days of incubation at 28°C, the plate was treated for 15 minutes with 0.1 % Congo red dye, followed by a 1 M NaCl solution. Clear yellow zones development indicates a positive result for cellulase production (19). Pectinase production was tested by combining pectin with M9 medium, 1 g $(NH_4)_2SO_4$, 1 g Dextrose, 10 g pectin, 0.5 g sodium citrate, 1.2 g yeast extract, 20 g agar (g L^{-1}). The formation of a clear zone when plates flooded with Lugol's iodine solution for 10 minutes after three days of incubation at 28°C denotes the isolate's ability to produce pectinase (32).

Ammonia and siderophore production

The bacterial isolates were inoculated in 10 ml peptone water for ammonia synthesis. After 48-72 h of incubation at 28 °C, the culture was centrifuged, and the supernatant loaded with 1 ml of freshly prepared Nessler's reagent, the positive test results a brown-yellow colour (34). Siderophore production was determined on a nutrient agar (NA) supplemented with chelating 2,2-Bipyridyl solution (2 mg L^{-1}), which filtered in NA at 45°C The growth of isolates signalled a positive test (31).

Indole acetic acid (IAA) production

IAA production was determined using Erlenmeyer flasks containing 50 ml of Nutrient broth medium (NB) with 0.2% (v/v) L- tryptophan. Flasks were inoculated with 2% of bacterial growth (0.5 O. D; 1×10^8 CFU ml^{-1}) and subjected to 120 rpm in a shaker incubator at 28°C for 48 h. 10 ml of growth sample was pipetted from each flask and centrifuged for 15 min at 10000 rpm. The supernatant was mixed with twice the volume of Salkowski'sr reagent (1 ml of 0.5M $FeCl_3$ and 49 ml of 35% $HClO_4$) and re-incubated for 30 min at 25°C in a dark place, then estimated calorimetrically at 530 nm (12).

Identification of bacterial isolates

Bacterial isolates were identified using the Vitek 2 compact system. The activated isolates, on MacConkey ager at 28°C for 24 hours, were used to prepare bacterial suspension by transferring to 3 ml of sterile saline. The turbidity was fixed at 0.5 OD using the McFarland standard. The kit was added for each tube and incubated for 18 hours in the device.

MIC determination

The bacterial isolates were subjected to $CuSO_4$, $CdCl_2$, and $ZnCl_2$ on Mueller-Hinton agar plate by a gradient concentration of heavy metals. The concentration level of heavy metals was started at 100 µg/ml and increased by 50 µg/ml, except for the $HgCl_2$ increased by 10 µg/ml, plates then incubated for 48 h at 28°C. MIC was determined on the plate medium when there was no visible growth (1)

DNA Extraction

DNA from the selected isolates were extracted using the ABIOpureTM Total DNA kit (ABIOpure, USA), according to the company protocol. A Quantus Fluorometer was used to quantify the nucleic acid concentration to detect the integrity of DNA for downstream applications. For DNA measurement, one µl of DNA was added to 199 µl of diluted QuantyFlour Dye and incubated at room temperature for five minutes.

Detection of heavy metal resistance genes

PCR test was used to detect heavy metal resistance genes using specific primers (Table 1) for amplifying the region 475bp

364bp and 206bp of *copA*, *copB*, and *czcA*, respectively. The PCR reaction was carried out in a 20µl volume, which was composed of 10µl master mix, 1µl forward primer, 1µl reverse primer, 2µl DNA template, and 6µl of nuclease-free water. The program used was illustrated in the Table 2. The PCR products were analysed on the agarose gel electrophoresis (1.5%, w/v), formed from 1X TAE buffer with 1µl of ethidium bromide (10mg/ml). The wells were loaded with PCR products (10µl), then powered at 100v/mAmp for 75 minutes. After that, the stained bands were displayed under the UV Transilluminator.

Table 1. Primers of heavy metals resistances genes.

Gene		Primer sequence 5′→3′	Annealing temperature	Product size
copA	F	CGGTCTCTACGAATACCGCTTCAA		
	R	GAAATAGCTCATTGCCGAGGCGTT	55°C	475bp
copB	F	TTCCTGCTCGACCAGTTGGAATAC		
	R	GGTTGGTCAACAGGATGTCGTACT	58°C	364bp
czcA	F	GTTCACCTTGCTCTTCGCCATGTT		
	R	ACAGGTTGCGGATGAAGGAGATCA	56°C	206bp

Table 2. PCR program for amplification heavy metals gene of *Pseudomonas aeruginosa*.

Steps	Temperature	Time (m:s)	Number of Cycle
Initial Denaturation	95°C	05:00	1
Denaturation	95°C	00:30	
Annealing *copA*	55°C	00:30	
Annealing *copB*	58°C	00:30	30
Annealing *czcA*	56°C	00:30	
Extension	72°C	00:30	
Final extension	72°C	07:00	1
Hold	10°C	10:00	

Statistical analysis

The data were statistically analyzed using ANOVA by the Statistical Analysis System program to compare the means of triplicate samples with the least significant difference (LSD) values (36).

RESULTS AND DISCUSSION

Isolation of *Pseudomonas* spp.

Bacterial isolates were gained from different rhizospheric soils, and characterized depending on morphological, cultural, and biochemical characteristics. The isolates were positive for oxidase, catalase tests, Simon citrate, and motile, while negative for Gram stain, indole, Voges-Proskauer, methyl red and starch hydrolysis. Accordingly, seventy isolates were mostly referred to *Pseudomonas* spp. (15).

Hydrolytic enzymes production

The result in Table 3 shows that the seventy isolates, 41 (58.57%) produced protease enzyme on the skimmed milk agar, with a zone varying between 2 and 14 mm. The highest was B50 (14 mm), followed by B55 (12 mm), while B32, B47, and B69, showed 10 mm in diameter. Only four (5.71%) isolates can produce cellulase in the CMC agar medium. The clear zone varies between 3 and 23 mm, the higher strain was B66 (23 mm) followed by B69 (20 mm) and B61 (13 mm). In addition, 15 (21.42%) isolates were pectinase producers in the M9 minimal medium. The zone of pectinase varied between (6-20 mm). B7 strain was the higher (20 mm), followed by B48 and B66 (15mm). Lytic enzymes can inhibit the growth or suppression of phytopathogens by degrading the fungal cell walls. Chitin and fibrils of glucan are incorporated into the protein matrix, that's why proteases play an essential role in fungus cell wall destruction (22). Alsalim (8) reported the production of hydrolytic enzymes by *Pseudomonas* spp. and found that all isolates were positive for protease and pectinase, while 66.6% of them could produce cellulase. Other researchers isolated 87 *Pseudomonas* species from the tomato (*Lycopersicon esculentum*) rhizosphere. They reported that only 30, 28, and 12 isolates were positive for protease, cellulase, and pectinase, respectively. Also found that the lytic enzymes in 30 isolates have antagonistic activity against the plant pathogenic bacterium *Ralstonia solanacearum* (39). Another study isolated three *Pseudomonas fluorescens* isolates from the plant rhizosphere, their results showed that the three strains were positive for protease, while negative for cellulase, chitinase, and glucanase (41)

Siderophore and ammonia production

In this study, the siderophore test was positive for all the isolates (Table 3). The seventy isolates revealed a growth on the 2,2 dipyridyl-containing agar media after 48 hours of incubation, which could contribute to their antifungal activity. The qualitative test of ammonia production in peptone water revealed that all isolates were positive, the colour changed to yellow or brown in peptone

water of old culture. In their study, Kotasthane *et al.* (24) mentioned that out of twenty-nine only eight isolates produced siderophores in the presence of 8-Hydroxyquinoline (50 mg/l). The eight isolates have antagonistic effects against *Rhizoctonia solani* and *Sclerotium rolfsii*. In another study, *P. asplenii, P. fluorescens,* and *P. aeruginosa* isolates showed a high level of inhibition against *Rhizoctonia solani*, 93.15%, 88.70%, and 86.85%, respectively, with the ability to synthesise siderophore and ammonia production ability of all the isolates (5). Agrawal *et al.* (2) reported that twenty-four fluorescent *Pseudomonas* isolates subjected to different siderophore assays were shown to produce siderophores on an iron-deficient succinate medium.

Rana *et al*. (34) reported that twenty *Pseudomonas fluorescent* isolates, collected from apple orchards, were exhibited a positive result in ammonia and siderophore synthesis.

Indole-3-acetic acid (IAA) production

The amount of indole-3-acetic acid produced by *Pseudomonas* spp. isolates, evaluated quantitively in NB broth, was ranged from 1.20±0.03 to 35.15±0.76 µg/ml (Table 3). IAA has a role in the extension, division, and differentiation of plant cells. IAA also promotes the growth of lateral roots and root hairs, which increases nutrient uptake by surfaces and may result in higher levels of nutrient absorption and dramatically improve the plant's shoot length (40). The highest IAA producing strain was B49 (35.15±0.76 µg/ml) followed by B46 (28.59±0.05 µg/ml) and B4 (26.98±0.42 µg/ml). Sandilya *et al*. (37) reported that eight *Pseudomonas* spp. isolates derived from castor rhizosphere showed IAA production in vitro ranged from 5.19 µg/ml to 27.84 µg/ml. The strain RTE4 of *Pseudomonas aeruginosa*, isolated from tea rhizosphere, was tested by Chopra *et al*. (13), and they found high indole acetic acid production (74.54 µg/ml) after seven days of incubation. Wadekar and Kagne (40) isolated 25 *Pseudomonas* spp. from the rhizosphere of soybean. Their results showed the highest IAA production, which reached 19.34 µg/ml, was by the strain S3 after 48 h of incubation on King's B. Akter *et al.* (5) reported that *P. asplenii* and *P. fluorescens* isolates collected from rice plants were shown to have a maximum IAA production reached 51 µg/ml and 52 µg/ml, respectively, while *P. aeruginosa* did not produce IAA. Ali *et al*. (7) found maximum IAA production by *P. aeruginosa* (8.95 µg/ml) during testing 12 bacterial isolates. Two isolates belonging to *P. aeruginosa* (JB and JC) were isolated from Solanum melongena and Capsicum annuum and synthesised IAA by 15.54 and 23.38 µg/ml, respectively (30).

Isolates identification

Based on the previous data, eight isolates (Table 3) were selected and identified by the Vitek 2 compact system. The results revealed that all belong to *Pseudomonas aeruginosa*.

Heavy metals resistance

The resistance profile to the heavy metals $CuSO_4$, $CdCl_2$, and $ZnCl_2$ for eight *P. aeruginosa* isolates, was carried out on Mueller-Hinton agar. The results in Table 4 demonstrate that MIC values to the Copper were extending from 800-3200 µg/ml. The *B49* strain has recorded a higher MIC value (3200 µg/ml) which differs significantly from other strains, followed by the *B46* strain (2800 µg/ml). The MIC values for Cadmium resistance were ranging from 600-1000 µg/ml and the strain *B66* recorded a higher MIC (1000 µg/ml) differs significantly from other strains. The resistance to Zinc, represented in the form of MIC values, was extending from1050-2600 µg/ml. The strain *B46* recorded the value 2600 µg/ml which differs significantly from the other strains. Metals resistances were appeared to be heterogeneous among *P. aeruginosa* isolates, and B49 has a higher resistance to copper sulphate and zinc chloride when compared to other bacterial isolates Ghaima *et al*. (17). found that the MIC rates of cadmium have been ranging from 600 to 900 mg/l for *P. aeruginosa* that isolated from Al-Dora agricultural soil, while the MIC for Copper and Cadmium was 3600 µg/ml for the strain KZ5 of *Pseudomonas aeruginosa*, according to Benhalima *et al*. (11). Akinbowale *et al*. (4) isolated 129 strains of *Pseudomonas* from different Australian farms. The majority of the isolates showed 800 µg/ml MIC values for Cd and Co, more values for Cu and Pb (1600 µg/ml), and the highest for Cr and Pb (3200

µg/ml), while MIC for Mn was more than 3200 µg/ml. *Pseudomonas aeruginosa* exhibited a resistance to Pb^{2+} Cu^{2+}, Cd^{2}, the minimum inhibitory concentration ranged between 100 and 500 ppm, while it was showed no resistance, no growth, in the presence of Ag^{2+} and Hg^{2+} (28). Malik and Aleem (26) isolated 96 strains of *Pseudomonas* spp. from soils, they noticed that most isolates had MIC values up to 3,200 µg/ml and some up to 1,600 µg/ml, and 91.6% of them were resistant to Pb^{2+} and Cu^{2+}, while 62.5% were resistant to Zn^{2+}. Another study by Imron *et al*. (21) mentioned that *P. aeruginosa* isolated from sanitary landfills exhibited MIC values of more than 20 mg l^{-1} for Zn, Mg Cd and Pb, using the disk diffusion method.

Table 3. Plant growth traits of *Pseudomonas* spp.

No.	*Prot (mm)	Cell. (mm)	Pect. (mm)	Sid.	NH_3	IAA (µg/ml)	No.	Pro. (mm)	Cell. (mm)	Pect. (mm)	Sid.	NH_3	IAA (µg/ml)
B1	-	-	-	+	+	12.92 ± 0.17	B36	8	-	-	+	+	-
B2	-	-	-	+	+	9.80 ± 0.08	B37	4	-	9	+	+	3.36 ± 0.03
B3	-	-	-	+	++	8.31 ± 0.19	B38	8	-	-	+	+	6.68 ± 0.15
B4	-	-	-	+	++	26.98 ± 0.42	B39	-	-	-	+	+	4.79 ± 0.02
B5	-	-	-	+	++	9.17 ± 0.12	B40	7	-	8	+	+	3.61 ± 0.07
B6	-	-	-	+	+	12.89 ± 0.15	B41	-	-	-	+	+	7.94 ± 0.21
B7	9	-	20	++	++	14.80 ± 0.89	B42	-	-	-	+	+	16.40 ± 0.46
B8	2	-	-	+	+	16.86± 0.43	B43	5	-	-	+	++	21.68 ± 0.51
B9	4	-	8	+	++	13.47 ± 0.37	B44	8	-	-	+	++	24.21 ± 0.62
B10	-	-	-	+	++	11.79 ± 0.23	B45	-	-	-	+	++	6.8 ± 0.0.05
B11	-	-	-	+	++	5.77 ± 0.06	B46	8	-	-	+	+++	28.59 ± 0.05
B12	-	-	-	+	++	1.76 ± 0.02	B47	10	-	-	++	+	3.52 ± 0.24
B13	-	-	-	++	++	14.12 ± 0.44	B48	8	-	15	+	++	4.41± 0.04
B14	-	-	-	+	+	6.47 ± 0.16	B49	6	-	12	++	++	35.15± 0.76
B15	-	-	-	+	+	1.20 ± 0.03	B50	14	-	8	++	++	2.85 ± 0.013
B16	-	-	-	+	+	6.95 ± 0.33	B51	9	-	-	+	+	5.57 ± 0.016
B17	-	-	-	+	+	-	B52	8	-	-	+	+	3.88 ± 0.02
B18	-	-	-	+	+	14.5 ± 0.15	B53	9	-	8	+	+	4.19 ± 0.04
B19	2	-	-	+	+	6.77 ± 0.20	B54	-	-	-	+	+	3.67 ± 0.02
B20	-	-	-	+	++	9.54 ± 0.16	B55	12	-	8	+	+	24.41 ± 0.57
B21	6	-	10	+	++	16.11 ± 0.26	B56	7	-	-	+	++	9.58 ± 0.19
B22	-	-	-	++	++	6.51 ± 0.08	B57	-	-	-	+	++	6.99 ± 0.11
B23	8	-	-	++	+	2.53 ± 0.04	B58	8	-	9	++	++	14.98 ± 0.67
B24	8	-	-	+	+	2.17 ± 0.16	B59	6	3	-	++	++	24.96 ± 0.30
B25	9	-	6	+++	+++	9.82 ± 0.15	B60	9	-	-	++	++	5.61 ± 0.03
B26	8	-	-	+	++	3.60 ± 0.04	B61	9	13	-	++	++	7.83 ± 0.19
B27	-	-	-	++	++	3.89 ± 0.08	B62	5	-	-	++	+	21.44 ± 0.2
B28	-	-	-	+	+	5.74±0.09	B63	8	-	-	+	++	11.43±0.56
B29	-	-	-	+	+	16.90 ± 0.27	B64	6	-	-	+	++	7.72 ± 0.10
B30	-	-	-	+	+	6.45 ± 0.13	B65	8	-	-	+	+	6.03 ± 0.10
B31	-	-	-	+	++	11.53 ± 0.43	B66	9	23	15	++	++	12.20 ± 0.19
B32	10	-	-	+	++	20.06 ± 0.57	B67	4	-	-	++	+	6.35 ±0.02
B33	-	-	-	+	++	8.55 ± 0.15	B68	6	-	-	+	+	8.32 ± 0.09
B34	8	-	-	+	+	14.91 ± 0.58	B69	10	20	11	++	++	4.59 ± 0.06
B35	9	-	13	+	+	5.76 ± 0.06	B70	8	-	-	+	+	11.57 ± 0.15
								3.981	4.077	3.649	1.0	1.0	7.441 **
			LSD value (P≤0.01).					**	**	**	NS	NS	

*** Prot., Protease (mm); Cell., Cellulase (mm); Pect., Pectinase (mm); Sid., Siderophore.; IAA, Indole-3-acetic acid**
****Siderophore and NH_3 : low +, medium ++, high +++;**

Table 4. The MIC of heavy metal in *Pseudomonas aeruginosa*

Isolate	$CuSO_4$ µg/ml	$CdCl_2$ µg/ml	$ZnCl_2$ µg/ml
P. aeruginosa B7	1800	950	1050
P. aeruginosa B25	2600	600	1800
P. aeruginosa B40	1600	850	2400
P. aeruginosa B46	2800	700	2250
P. aeruginosa B49	3200	900	2600
P. aeruginosa B50	2200	850	1250
P. aeruginosa B66	2700	1000	1850
P. aeruginosa B69	800	600	1100
LSD value (P≤0.01).	271.48	84.52	163.87

Detection of heavy metals resistance gene in *Pseudomonas aeruginosa*

The eight studied *P. aeruginosa* isolates were subjected to PCR amplification to detect the presence of the genes responsible for their resistance to Cu, Cd, and Zn, *copA*, and *copB* genes for Cu and *czcA* gene for Cd, and Zn. The *copB* and *czcA* genes were observed in all eight isolates, while the *copA* gene was observed in only seven isolates (Figure 1). The *copA* and *copB* genes' presence were confirmed by many researchers in *P.*

aeruginosa that showed heavy metal resistance. Martins *et al.* (27) isolated two tetracycline-resistant strains of *P. aeruginosa* (EW32 and EW33) from a polluted environment and detected *copA, copB* and *czcA* genes presence using PCR. They said that treatment of numerous pathogenic bacteria in humans and animals can be limited due to the exchange of resistance genes between environmental and pathogenic bacteria. Their study observed that the EW32 strain was positive for *copA*, *copB* and *czcA* genes, while the EW33 strain was positive only for the *czcA* gene. Pitondo- Silva *et al*. (33) detected the occurrence of the genes in sixty-four *P. aeruginosa* that were isolated from different crops of five Brazilian regions. Their study showed that the most common existence of heavy metals resistance genes for *copB, copA* and *czcA* were 65%, 48% and 46%, respectively. Li *et al*. (25) mentioned that *copA* and lipoprotein had a role in the sequestration and efflux of copper out of the cytoplasm and proposed a response model for *Pseudomonas* spp. under higher concentrations of copper. They noted the reduction in cell size, which leads to reducing the quantity of copper bonded around the cell surface, and less energy had required to maintain themselves during copper stress.

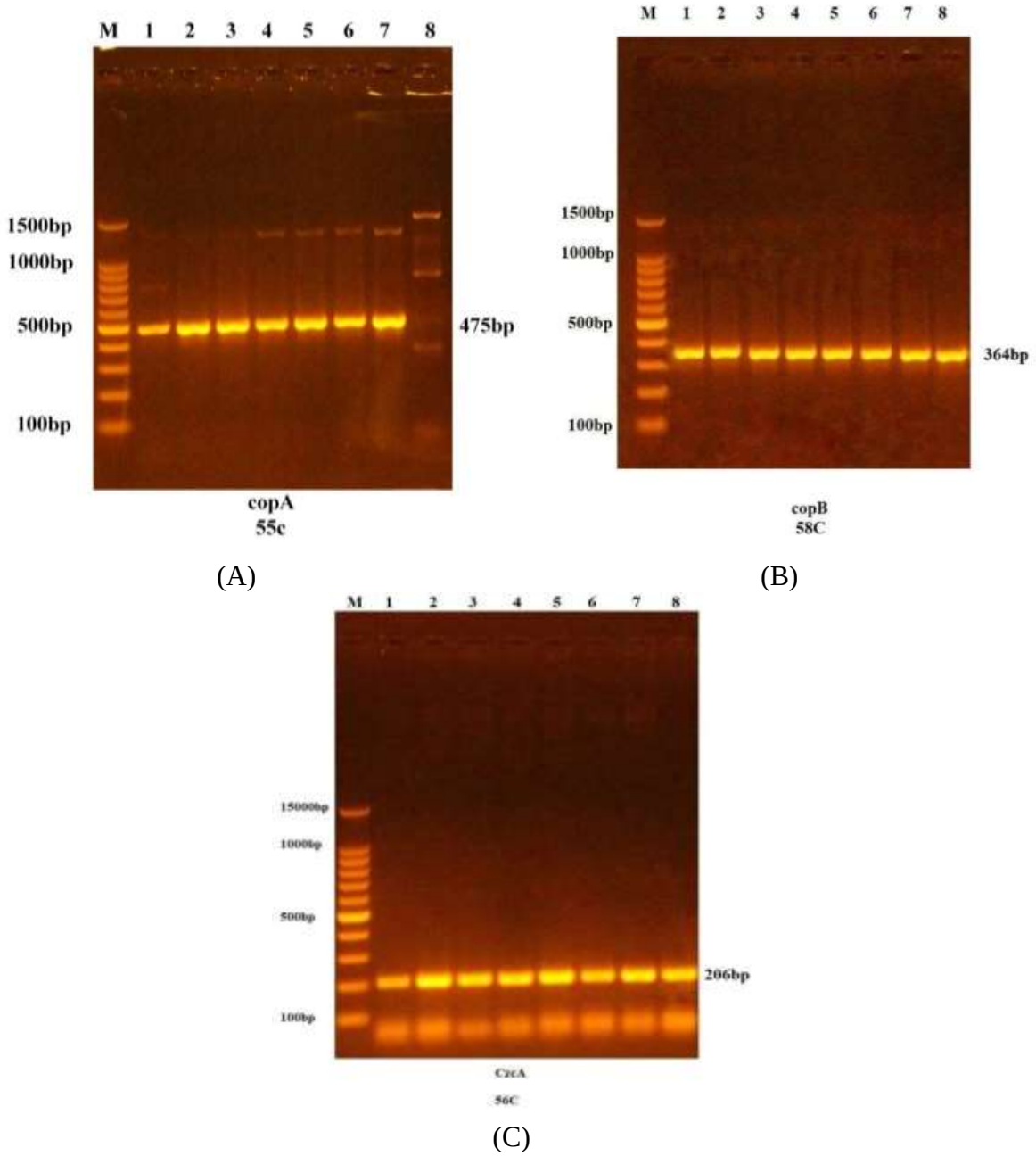

Figure 1. (A), (B) and (C) showed amplification products of *copA* (475bp), *copB* (364bp) and *czcA* (206bp) of *P. aeruginosa* isolates in 1X-TAE buffer and 1.5% Agarose gel.

CONCLUSION

Results indicate that several *Pseudomonas* spp. isolated from rhizospheric soil showed many plant growth promotion traits. These traits were protease, cellulase, pectinase, siderophore, ammonia, and IAA production. They are offering an alternative fertilizer (biofertilizer) and pesticide (biopesticide), as they exhibited these traits. Based on this study's obtained data, the eight *P. aeruginosa* strains have varied resistance to the examined heavy metals, as they showed high MIC values of resistance for Cu, Cd, and Zn. The detection of *copA*, *copB*, and *czcA* genes, which play a role in the efflux pump of heavy metals, approved the genes' presence. *P. aeruginosa* can be promising candidates for bioremediation of polluted soils.

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