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Evaluation of Bacteria from Soil and Rhizosphere as Herbicidal Candidates of Some Broadleaf Weeds

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THE USE of bacteria in biological weed control is an alternative ecofriendly way to tackle weed problems and reduce the risk of herbicide resistance. In this study, we obtained four main active bacterial isolates under the genera *Pseudomonas, Bacillus* and *Xanthomonas* from Wadi El Natroun region. All the tested bacterial isolates caused high significant reductions in seed germination and seedling growth of *Convolvulus arvensis* and *Portulaca oleracea*. Bioassaying ethyl acetate crude extracts of these isolates showed that *Pseudomonas* sp. (isolate 1) was the most active against seedling stage of *Portulaca oleracea*. The cultural filtrate of the same isolate caused 100% reduction when assayed on seed germination, shoot and root length of *Convolvulus arvensis* and *Portulaca oleracea.* At the highest concentration (40mg/ml) of the crude ethyl acetate extract, the reduction percentage in total biomass fresh weights of *Portulaca oleracea* and *Convolvulus arvensis* seedlings reached 71.27 and 39.37%, respectively. The EC_{50} values (concentration that inhibited growth by 50%) were 1.3 and 1.64mg/ml for *Portulaca oleracea and Convolvulus arvensis,* respectively. Comparative 16S rRNA gene sequencing analysis and biochemical characterization demonstrated that isolate FS15 was member of the genus *Pseudomonas* and belongs to the *Pseudomonas aeruginosa* group with *Pseudomonas aeruginosa* strain NR 113599.1 as the closest relative (99.5% sequence similarity). In conclusion, *Pseudomonas aeruginosa* has high potential to be developed as natural bacterial herbicide and may be used in broadleaf weed control.

Keywords: Rhizobacteria, Biocontrol agent, *Pseudomonas aeruginosa,* Noxious weeds, Bioherbicides.

Introduction

Weeds are unwanted plants that compete with crop plants for space, nutrients, water, sunlight, and other elements. They are under estimated crop pests causing approximately 37% losses in the yields of crops **(**Sindhu & Sehrawat, 2017 and Ferreira & Reinhardt, 2016). According to Holm et al. (1991) *Convolvulus arvensis* is one of the world's top noxious weeds and it is found in 32 different crops in 54 countries. Meanwhile, *Portulaca oleracea* is listed as a very aggressive noxious weed infesting gardens, wetlands, lawns and agricultural areas (Smith & Figueiredo, 2010) and has been ranked the eighth most common weed in the world (Moneim et al., 2013).

Weed management in recent decades is changing from conventional practices to the use of environment friendly classical biological and bioherbicidal weed control approaches in crop production (Javaid, 2010 and Javaid & Ali, 2011). Interest in developing effective biological weed management systems continues to increase because of a growing awareness of problems associated with the constant and intensive use of chemical herbicides. Most herbicides cause some visible stress to crops and non-target organisms, damage surface and ground water, raise resistance in weeds, capable of entering the body via food chain threating human and animal health (Gliessman, 2002 and Kremer, 2006). Microbial based weed control of which soil microorganisms is part represents an innovative means to manage troublesome weeds. Application of naturally occurring plant suppressive microbes that are found in soil has recently received considerable attention, as a promising approach for controlling weeds by providing healthy/ economically sounder herbicides and a limited chance of developing herbicides-resistant weeds

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(Weissmann & Gerhardson, 2001; Omer et al., 2010 and Radhakrishnan et al., 2016).

Rhizosphere microorganisms have been found to suppress the growth of weeds by reducing plant density, biomass and seed production. Bacteria produce a wide array of phytotoxins that may cause mortality of weed plants (Sindhu & Sehrawat, 2017). The object of deleterious rhizobacteria is not to completely kill or eradicate the weed population; but rather to reduce the competitive pressure of the weed leading to decrease in their biomass accumulation and reduction in seed production (Kremer, 2006). Utilizing rhizospheric bacterial isolates to inhibit growth of weeds but not that of crop plants will benefit agriculture by contributing to increased crop yields and reducing weed competition (Patil, 2014).

Biological control is still extremely limited in weed control applications; this study focuses on using bacteria which received less attention than using fungi in controlling broad-leaved weeds. The study aimed to increase the biological sources of bioherbicides by screening rhizospheric and soil bacteria that have potential herbicidal activity using *Convolvulus arvensis* and *Portulaca oleracea* as test organisms.

Materials and Methods

Bacterial isolation from soil

Bacteria were isolated from nine types of rhizosphere and non-rhizosphere soils (bulk soil) of *Convolvulus arvensis*, *Typha* sp., *Melilotus indica*, *Cortaderia selloana*, *Echinochloa colonum*, *Cynodon* sp., *Malva* sp., *Chenopodium* sp. and *Cyperus rotundus* at Wadi El Natroun region, Egypt during January 2016. After collection, soil samples were kept in refrigerator at 4°C until investigation. The isolation of bacteria was processed by serial dilution method according to Johnston & Booth (1983). Pure bacterial culture colonies were maintained on nutrient agar (NA) slants at 4ºC for testing further studies.

Effect of bacterial culture filtrate on Convolvulus arvensis and Portulaca oleracea seeds

The cell free culture filtrate of bacteria was prepared by culturing bacterial isolates on NA medium and incubated at 30ºC for 24-48hr according to Landa et al. (1997). A suspension of each bacterial isolate was prepared and inoculated into a 250ml Erlenmeyer flask containing 100ml nutrient broth (NB) in a shaking incubator (Daihan scientific WIS-30) at 120rpm and 30°C for two days. The broth was centrifuged under cooling to remove bacterial cells then, the supernatant was filtered through sterile 0.45µm pore size Millipore filters (Millex syring filter). All supernatants were collected and stored at 4ºC until bioassay tests. The seeds of *Convolvulus arvensis* and *Portulaca oleracea* were surface sterilized by soaking in 0.5% sodium hypochlorite (NaOCl) for 2min followed by rinsing thoroughly in sterile distilled water and plotted on filter paper then drying under a laminar flow hood. The sterilized seeds (5 seeds per dish) were sprinkled in sterile 9cm diameter petri dishes containing sterile filter paper and 5ml of cultural filtrate was added under aseptic conditions. Controls were inoculated with 5ml of non-inoculated sterile media. Petri dishes were sealed with parafilm and incubated in darkness at 25±2°C. Three replicates were used for each treatment. The germination and seedling (root and shoot length) responses were determined 7 days after incubation.

Preliminary identification of active bacterial isolates

Bacterial isolates that inhibited the target weeds under laboratory conditions were morphologically characterized using standard microbiological methods for Gram-reaction to differentiate G +ve from G -ve bacteria and then biochemically characterized using motility, catalase, urease, citrate, nitrate reduction and oxidase production (Prescot et al., 1993).

Bioassay of bacterial crude extracts on Convolvulus arvensis and Portulaca oleracea seedlings

Active culture filtrates were adjusted to pH 3.8 then extracted 3 times with an equal volume of ethyl acetate. Extracts were then completely dried using a rotary evaporator to give crude oily residues, which were re-dissolved in 10% ethanol from which a series of concentrations (5, 10, 20, 30, 40mg/ml) were prepared. Prepared concentrations were added into tissue culture tubes containing 3ml of liquid Murashige & Skoog medium (MS medium) where the roots of seven days old seedlings were submerged (one seedling per tube). Three replicates were used for each concentration. After incubation at 25°C

for seven days, the seedlings were removed, biomass fresh weights were measured and the reduction percentage (R %) was calculated (Balah, 2012).

Molecular identification of the most active isolate

The 16S rDNA gene analysis of the most active isolate was carried out using MicroSeq 500 system. Amplification of 500bp of 16S rRNA gene using GeneAmp 9600 thermocycler (Applied Biosystems). Amplicon detection was verified by 2% agarose gel electrophoresis previous purification with YM-100 Microconcentrators (Celera Diagnostics). The sequencing reactions were performed with MicroSeq500 16S rDNA. Bacterial Identification Sequencing kit, cycle reactions were purified using Centri-Sep Spin Columns (Applied Biosystems) and the cycle sequencing products were analysed on 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Sequences from both strands were aligned by using NCBI BLAST 2 Sequence. Then phylogenetic tree was constructed according to Saitou &Nei (1987) using MEGA 5.0 Molecular Evolutionary Genetics Analysis software version 5 (Tamura et al., 2011).

Statistical analysis of results

Data of Randomized Complete Block Design experiments were statistically analyzed by ANOVA according to Snedecor & Cochran (1990) and treatment means were compared by LSD test at 5% level of probability using CoStat. The EC_{50} values (half maximal effective concentration) for growth parameter were calculated by plotting concentration on a log scale (X) and the response (Y) on probit scale mathematically transformed, the data appear linear and sign the point in a semi-log graph paper.

Results

Herbicidal activity of bacterial isolates culture filtrate

Screening of a total of 40 bacterial isolates on seed germination of *Convolvulus arvensis* and *Portulaca oleracea* lead to four main active isolates (FS15, PS, BRS2, and BS47). The selection of these four active isolates was based on reduction percentage of germination, shoot length, and root length over 70%, the results of the rest of isolates was not reported. The source and isolation site of the selected bacteria are shown in Table 1. The application of four bacterial culture filtrates on *Convolvulus arvensis* seeds resulted in a complete inhibition on the germination (%) compared to the untreated control concurrently with a significant reduction in length of shoot and root. While, in case of *Portulaca oleracea*, 3 isolates (FS15, PS and BS47) gave 100% reduction in germination, shoot length and root length while the fourth isolate BRS2 gave 75% inhibition in seed germination and 89.34%, and 73.68% reduction in shoot and root length, respectively as shown in Table 1.

TABLE 1. Source and location of active bacterial isolates and their effect on *Convolvulus arvensis* and *Portulaca oleracea*.

Bacterial isolate code	Source of isolation	Location	Reduction % on *Convolvulus arvensis*			Reduction % on *Portulaca oleracea*		
			G	SL	RL	G	SL	RL
FS15	Soil of *Chenopodium* sp.	Natural vegetation	++	++	++	++	++	++
PS	Soil of *Convolvulus arvensis*	Sugar beet farm	++	++	++	++	++	++
BRS2	Rhizosphere of *Cynodon* sp.	Sugar beet farm	++	++	++	+	+	+
BS47	Soil of *Cortaderia selloana*	Grapes farm	++	++	++	++	++	++

G= Germination, SL= Shoot length, RL= Root length.
(++) Represents 100% reduction, (+) means reduction more than 70%.

Preliminary identification of main active bacterial isolates

Preliminary morphologically identification of the 4 selected isolates showed FS15, PS and BS47 are gram negative bacilli while BRS2 gram positive bacilli. Biochemical tests demonstrated that 2 isolates (FS15 and PS) were members of the genus *Pseudomonas* whereas the other two (BS21 and BS47) were shown to belong to the genus *Bacillus* and *Xanthomonas* sp., respectively (Table 2).

Herbicidal potential of bacterial crude extracts on weed seedlings

The bioassay with ethyl acetate crude extracts of the four bacterial isolates showed that *Pseudomonas* sp. (isolate 1) gave the highest significant reduction in total biomass fresh weight of *Portulac oleracea* seedlings, regardless the concentration tested. At a concentration of 5, 10, 20, 30, and 40mg/ml crude extract, the reduction percentage was 16.00, 17.46, 34.55, 46.91 and 71.27, respectively as shown in Table 3. The second highly active extract was that of *Pseudomonas* sp. (isolate 2) which gave 51% reduction at 40mg/ml. Table 4 is summarizing the total biomass fresh weight of *Convolvulus arvensis* in response to applying bacterial crude extracts. The highly active extract was that of *Xanthomonas* sp. which gave 40.34% reduction in fresh weight at the highest concentration 40mg/ml, followed by *Pseudomonas* sp. (isolate 1) which gave 39.37% reduction. Also, at 40mg/ml *Bacillus* sp. and *Pseudomonas* sp. (isolate 2) gave 38.36 and 35.58% reduction in biomass fresh weight.

TABLE 2. Biochemical tests of active bacterial isolates.

	FS15	PS	BS47	BRS2
Gram stain	-	-	-	+
Urease	-	+	-	-
Citrate	+	+	-	+
Catalase	+	+	+	+
Starch hydrolysis	-	-	+	+
Nitrate reduction	+	+	-	+
TSI	-	-	+	-
MR-VP	-	-	-	-
KOH	-	-	+	-
Glucose oxidase	+	+	-	-

TABLE 3. Effect of bacterial ethyl acetate crude extracts on total biomass fresh weight (mg) of *Portulaca oleracea.*

Isolates \ Concentration (mg/ml)		Control	5	10	20	30	40	LSD (0.05)
Pseudomonas sp. isolate 1	Mean	18.00^{a}	16.00^{ab}	16.00^{ab}	12.00^{bc}	10.00^{cd}	6.00^{d}	2.39
	R%	0.00	16.00	17.46	34.55	46.91	71.27	
Pseudomonas sp. isolate 2	Mean	16.00^{a}	20.00^{b}	18.00^{bc}	16.00^{bc}	12.00^{c}	8.00^{d}	1.74
	R%	0.00	-24.45	-11.35	0.87	15.72	51.10	
Bacillus sp.	Mean	27.00^{a}	28.00^{a}	27.00^{a}	23.00^{b}	20.00^{c}	18.00^{c}	3.18
	R%	0.00	-3.42	2.19	14.76	26.71	33.17	
Xanthomonas sp.	Mean	27.00^{a}	36.00^{ab}	33.00^{bc}	29.00^{c}	21.00^{d}	17.00^{d}	5.35
	R%	0.00	-31.95	-20.12	-6.58	21.71	39.39	

Mean= Average values after seven days, R%= rReduction percentage in biomass fresh weight, LSD= Lleast significant difference.
Values with the same letter in each column do not differ from each other statistically (P≤ 0.05).

TABLE 4. Effect of bacterial ethyl acetate crude extracts on total biomass fresh weight (mg) of *Convolvulus arvensis*.

Isolates \ Concentration (mg/ml)		Control	5	10	20	30	40	LSD (0.05)
Pseudomonas sp. isolate 1	Mean	147.00^{a}	125.00^{b}	116.00^{c}	109.00^{c}	101.00^{cd}	89.00^{d}	19.18
	R%	0.00	15.38	21.27	26.02	31.45	39.37	
Pseudomonas sp. isolate 2	Mean	238.00^{a}	190.00^{b}	183.00^{b}	172.00^{c}	163.00^{cd}	153.00^{d}	9.55
	R%	0.00	20.00	23.16	27.79	31.58	35.58	
Bacillus sp.	Mean	219.00^{a}	222.00^{a}	197.00^{b}	173.00^{c}	147.00^{d}	135.00^{d}	15.04
	R%	0.00	-1.37	9.89	20.85	32.73	38.36	
Xanthomonas sp.	Mean	110.00^{a}	117.00^{a}	106.00^{a}	81.00^{b}	79.00^{b}	65.00^{b}	17.89
	R%	0.00	-6.54	3.50	25.99	28.25	40.34	

Quantitive assessment of effective concentration (EC_{50})

The median effective concentration (EC_{50}) was determined as shown in Table 5 from probit curves, while the linear line was generated. Based on EC_{50} values *Pseudomonas* sp. isolate 1 showed the lowest value when compared to the other isolates. The (EC_{50}) of this isolate was determined on *Portulaca oleracea* (total biomass fresh weight parameter) by 1.3mg/ml. The dose response curve of *Pseudomonas* sp. isolate 1 was shown in Fig. 1.

Identification of the most active isolate by molecular technique

The most potent isolate was identified by 16S rDNA gene sequence. The bacterial isolate 99% identical to *Pseudomonas aeruginosa*. The phylogenetic tree of the isolate with the nearest strains revealed that it was closely related to *Pseudomonas aeruginosa* strain NR 113599.1 (Fig. 2).

TABLE 5. Effective concentration (EC_{50}) of microbial crude extracts on total biomass fresh weight of weed seedlings.

Isolates	EC_{50} (mg/ml)	
	Convolvulus arvensis	***Portulaca oleracea***
Pseudomonas sp. isolate 1	1.64	1.30
Pseudomonas sp. isolate 2	N.D	1.63
Bacillus sp.	1.79	N.D
Xanthomonas sp.	1.69	1.72

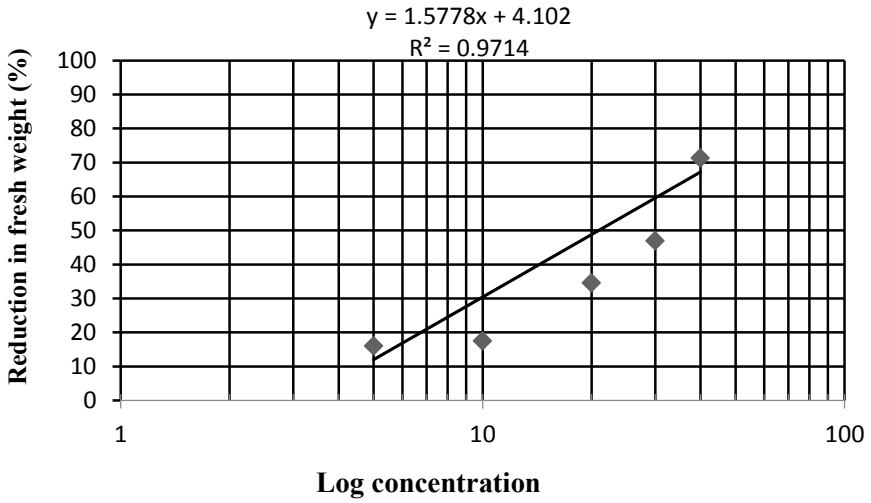

Fig. 1. Dose response curve of *Pseudomonas aeruginosa* on *Portulaca oleracea* total biomass fresh weight.

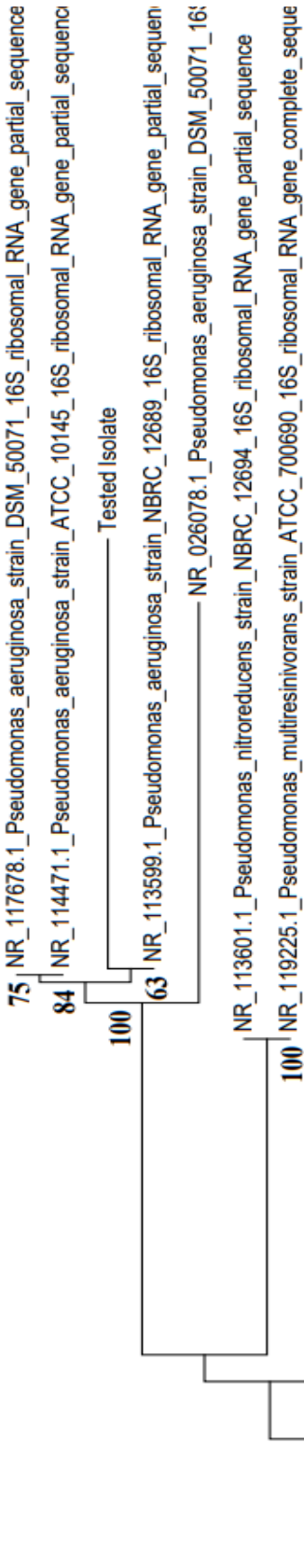

Fig. 2. Neighbour-joining phylogenetic tree showing the relationships between tested isolate and related taxa by MEGA program.

Discussion

Weed is a concern in both agriculture and ecology and scientists are always testing new natural herbicides in order to improve crop yields and eradicate invasive species without damaging the environment. The ability of bacteria to inhibit growth of various weed taxa in different cropping systems is well documented (Kremer & Souissi, 2001 and Patil, 2013). In this study, the herbicidal activity of 40 bacterial isolates was evaluated on *Portulaca oleracea* and *Convolvulus arvensis* of which four bacterial isolates gave the highest herbicidal effect on seed germination and seedling growth. Preliminary identification based on morphological features and biochemical characterization revealed that two, designated as isolate 1 and isolate 2, were belong to the genus *Pseudomonas*, meanwhile the other two were members of the genus *Bacillus* and *Xanthomonas*. Based on seedling growth bioassay, we identified the high active one through gene sequencing as *Pseudomonas aeruginosa*. A dose-based activity was reported with highly significant results against *Portulaca oleracea* rather than *Convolvulus arvensis*.

In our study, we also reported a high characteristic herbicidal activity of *Bacillus* sp. and *Xanthomonas* sp. on *Portulaca oleracea* and *Convolvulus arvensis* seeds. There are many assumptions in the literature of the potential herbicidal activity of *Bacillus* and *Xanthomonas* sp. based on isolate screening and biological assessments. *Pseudomonas aeruginosa* has been also alleged to be a source of biologically active agents with high herbicidal activity (Hadizadeh et al., 2014 and Boyette & Hoagland, 2015). The potential activity of three rhizomicroorganisms (*Pseudomonas syringae* st.1*, Pseudomonas syringae* st.2 and *Colletotrichum* sp.) was reported against *Polyogon monspeliensis, Phalaris paradox* and *Convolvulus arvensis*. In this context the ethyl acetate extracts of *P. syringae* st.2 and *Colletotrichum* sp. showed inhibitory effects at 120µg ml^{-1} with a decline in seedling growth reached maximal values. Meanwhile, *C. arvensis* was slightly affected by most treatments except for *P. syringae* st.1 which reduced seedling biomass by 22.83% at 120µg ml^{-1} (Omer & Balah, 2011). Lakshmi et al. (2015) used *Pseudomonas aeruginosa* KC1 in a form of bacterial suspension to inhibit seedling growth of *Portulaca oleracea* both *in vitro* and in glasshouse studies where it caused a high reduction in root length and biomass. To a large extent, this is in line with results of our study; treatment with culture filtrate of *Pseudomonas aeruginosa* resulted in 100% reduction on seed germination, shoot and root length of *Portulaca oleracea* while, using the crude ethyl acetate extracts resulted in 71.27% significant reduction in total biomass fresh weight of seedlings at 40mg/ml. In this context, Adetunji et al. (2017) proved that the crude extract of *Psudomonas aeruginosa* had high necrotic activity against *Amaranthus hybridus* L. and *Echinochloa crus galli* (L.) Beauv.

The chemical nature of microbe-derived natural products is as varied as the microorganisms themselves (De Luna et al., 2011) which range from simple compounds like cyanide and organic acids (Kremer & Souissi, 2001) to complex compounds like secondary metabolites (Kroschel & Elzein, 2004) including plant growth regulators such as auxins and ethylene (De Luna et al., 2005). For the well-studied biological activities, the negative impacts of *Pseudomonas aeruginosa* against weeds is associated with the production of Hydrogen Cyanide which result in lethality in most cases (Kremer & Souissi, 2001). HCN inhibits the terminal cytochrome c oxidase in the respiratory chain and binds to metalloenzymes and may have deleterious effects on several plants (Jain & Das, 2016).

Conclusion

The use of microbial metabolites produced by *Pseudomonas aeruginosa* could be promising for the management of weeds in conventional and organic farming to improve yield and enhance food security. Even though the current study gave special attention to assess both cultural filtrates and crude ethyl acetate extracts on two of the most aggressive weeds in the Egyptian agricultural community, further study will be needed to explore the responsible phytotoxins and screening activity under large-scale programs including the effect on non-target organisms and stability under various environmental conditions.

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تقييم للبكتريا المعزولة من التربة والمناطق الجذر محيطية كمبيدات عشبية لبعض الأعشاب عريضة الأوراق

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يعتبر استخدام البكتريا فى المكافحة الحيوية للحشائش بديل صديق للبيئة للقضاء على مشكلة الحشائش و تقليل مخاطر حدوث مقاومة للمبيدات. فى هذه الدراسة تم الحصول على أربع عزلات بكتيرية فعالة من منطقة وادى النطرون تنتمي لأجناس *Pseudomonas*, *Bacillus*, *Xanthomonas*. جميع العزلات البكتيرية المختبرة أحدثت نسب خفض عالية فى إنبات و نمو بذور كل من الرجلة و العليق. و كان مستخلص الإيثيل أسيتات لإحدى العزلات المنتمية لجنس *Pseudomona* هو الأكثر فاعلية ضد بادرات الرجلة. علاوة على ذلك، فقد أدت هذه العزلة إلى خفض انبات و نمو بذور الرجلة و العليق بنسبة 100%. وعند أعلى تركيز من مستخلص الإيثيل أسيتات (40ملجم/مل) كانت نسبة الخفض فى الكتلة الحيوية لبادرات الرجلة و العليق 71.27 و 39.37% على التوالى. و تم تعيين قيمة التركيز النصفى المؤثر (EC_{50}) لكل من الرجلة و العليق و كانت 1.3 و 1.64 ملجم/مل، على التوالى. أوضحت الاختبارات البيوكميائية و كذلك التعريف الجزيئي للعزلة الأكثر فاعلية أنها تنتمى إلى نوع *Pseudomonas aeruginosa* و أن اقرب سلالة لها في بنك الجينات هى *Pseudomonas aeruginosa* strain NR 113599.1 بنسبة تشابه 99.5%. واستناداً للنتائج المستخلصة من الدراسة يمكننا استنتاج أن عزلة *Pseudomonas aeruginosa* السابق ذكرها يمكن تطويرها إلى مبيد عشبى طبيعى قادر على مكافحة بعض الحشائش العريضة.