



Article

Isolation, Characterization and Herbicidal Activity of *Pseudomonas fluorescens* for Controlling Some Wheat Weeds

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Abstract

Pseudomonas spp was isolated from the rhizosphere area of two wild plants Radish *Raphanus raphanistrum* and barley *Hordeum spontaneum* naturally grown in a wheat *Triticum aestivum* field. The inhibitory activity of this bacterium was tested against the germination of weed seeds. Six isolates of *Pseudomonas* sp. were recovered from symptomatic weed plants exhibiting stunting and yellowing that collected from different locations at Baghdad Province. The isolates of *Pseudomonas* spp varied in their inhibitory activity against seed germination of wild radish and barley, scoring (8.75-92.5) and (10.00-96.25), respectively. The isolates PR4 and PR6 maximum inhibition rates on wild radish and barley ranged (82.50-92.50) and (90.00-96.25), respectively. Whilst, bacterial filtrates when applied could inhibit the seed germination of the two weeds scoring (6.25-90.00) and (5.00-93.75), respectively. Amongst other isolates, filtrates from PR4 and PR6 scored the highest inhibition rates in the seed germination of wild radish and barley which ranged (85.00-90.00) and (88.75-93.75) respectively. Based on morphology and molecular identification PR6 and PR4 were bacterial isolates of *Pseudomonas fluorescens*. Nucleotide sequences of the two isolates were deposit in the NCBI GenBank data base under the accession numbers MW740144 and MW740145. Data indicated the application of bacteria and their filtrates enabled an ecofriendly control against weeds.

Keywords: wheat, weed, *Pseudomonas fluorescens*, bioherbicide.

عزل وتوصيف لبكتيريا *Pseudomonas fluorescens* وفعاليتها لمكافحة بعض أنواع ادغال الحنطة

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المستخلص

عُزلت بكتيريا من جنس *Pseudomonas* من منطقة جذور نباتين بريين، هما الفجلية (*Raphanus raphanistrum*) والشعير البري (*Hordeum spontaneum*)، اللذين ظهرا طبيعيًا في حقل الحنطة (*Triticum aestivum*). واخُتبرت فعالية هذه البكتيريا في تثبيط إنبات بذور الأدغال. تم الحصول على ست عزلات من بكتيريا *Pseudomonas* من نباتات أدغال

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مصابة بأعراض التقزم والاصفرار، جُمعت من مواقع مختلفة في محافظة بغداد. تفاوتت فعالية عزلات *Pseudomonas* في تثبيط إنبات بذور الفجيلة والشعير البري، إذ تراوحت قيم التثبيط بين 8.75 و 92.5 و 10.00 و 96.25 على التوالي. أما أعلى معدلات التثبيط للعزلتين PR4 و PR6 على الفجيلة والشعير البري، فتراوحت بين 82.50 و 92.50 و 90.00 و 96.25 على التوالي. في حين أن راشح البكتيريا، عند استخدامه، مكن من تثبيط إنبات بذور نوعي الأدغال المختبرين، إذ سجلت (6.25-90.00) و (5.00-93.75) على التوالي. ومن بين العزلات الأخرى، سجل راشحا العزلتين PR4 و PR6 أعلى معدلات تثبيط في إنبات بذور الفجيلة والشعير البري، إذ تراوحت (85.00-90.00) و (88.75-93.75) على التوالي. وبناءً على التشخيص المظهري والجزيئي، تبين أن PR4 و PR6 عزلتان بكتيريتان من نوع *Pseudomonas fluorescens*. وقد تم إيداع تسلسلات النيوكليوتيدات للعزلتين في قاعدة بيانات NCI GenBank تحت رقمي الوصول MW740144 و MW740145. وتشير البيانات إلى أن استخدام البكتيريا ورواشحها يتيح مكافحة صديقة للبيئة ضد الأدغال.

1. Introduction

The wheat crops. *Triticum aestivum* L. belonged to the family Poaceae that is known as the king of cereals, which is an important food crop (1), and its original homeland is in northern Iraq. Wheat accompanies various species of weed, as more than 16 species of broad-leaf weed are spread in Iraq, including *Beta vulgaris*, *Ammi majus*, *Malva rotundifolia*, *Chenopodium album*, *Brassica nigra*, *Sonchus oleraceus*, *Raphanus raphanistrum*, *Convolvulus arvensis*, *Silybum marianum*, and *Cardoria draba*. Besides, there are more than 12 species of narrow-leaf weed including *Lolium rigidum*, *Avena Fatua*, *Lolium temulentum*, *Phalaris minor*, *Hordeum glaucum*, and *Cynodon dactylon* (2, 3). The tendency towards alternatives to weeds control has been implemented, as the microorganisms were exploited for biological control. Thus, a group of bacteria with a harmful and inhibitory effect on root growth, Deleterious rhizobacteria (DRB) was identified, because of its action in opposition to the promoting-growth rhizobacteria (PGPR) (4). Among the most important of these are the Species *P. fluorescens* and *Xanthomonas campestris* (5). It was found that *P. fluorescens* inhibits the growth of some species of weed or it inhibits seed germination as indicated by (5, 6). Strain D7 that isolated from the area around the roots of wheat (*Triticum aestivum*) and brome downy (*Bromus tectorum*) in western Canada has a significant effect on seed germination and growth of many weeds of the Poaceae family (5, 7). The specialized effect of this strain is due to its secretion of several peptides and lipopolysaccharide that were identified, are work together to provide the effect that responsible for its actions as a herbicide for this weed (8). It was also found that the strain WH6 of *P. fluorescens* affected the germination of a wide range of weed plants (21 species of monocotyledons and 8 species of dicotyledons, except for *Zea mays*) (9). The active compound responsible for this effect was identified using Mass spectrometry and found to be L-vinylglycine 4-formlaminooxy- (10). It has been suggested that the biosynthesis of this compound begins with the amino acid homoserine (11). Moreover, Other compounds with toxic effect were also identified in another strain of *P. fluorescens*, BRG100, which affected the growth of foxtail (*Setaria viridis*), Pseudophomin B and A, using High-Performance Liquid Chromatography (HPLC) and Thin Layer chromatography (TLC) techniques (5). This strain has been commercially produced and has proven its ability as a biocide (12). Also, the effect of *P. trivialis* strain X33d was tested against the weed *Bromus diandrus* in wheat cultivation fields in Tunisia, where a Turkespin was prepared for testing as a biocide for the weed control and it gave high efficiency in preventing the germination of the targeted weed seeds. It was also evaluated after storing for six months at a temperature of 4 °C (13). The addition of these types of bacteria to the soil led to inhibiting the growth of different types of weeds

including *Bromus tectorum*, *B. japonicum*, and *Aegilops cylindrical* (14, 15). The use of two strains (LS102 and LS174) of *Pseudomonas fluorescens* inhibited the roots of leafy spurge (*Euphorbia esula*) (9, 16). Furthermore, the BRG100 strain of *P. fluorescens* was used in a formulation of a commercial product called (Pesta) to inhibit foxtail *Setaria Viridis* (17). On the other hand, the G2-11 strain of *P. fluorescens* demonstrated considerable potential for inhibiting the roots growth of Velvetleaf weed in Canada (18). Data showed that *P. fluorescens* has significant ability to reduced weed competition and increased phosphorus availability in the soil (19). Therefore, the study aimed to isolate and identification of *Pseudomonas* spp. from the morphological and molecular soils around the roots of the wheat weed. Besides, testing the efficacy of *Pseudomonas* sp. in inhibiting the germination of weed.

2. Materials and Methods

2.1 Sample collection

Samples of weed plants (wild barley), *Hordeum vulgare*, (wild radish), *Raphanus raphanistrum*, which showed disease symptoms such as yellowing, stunting, and abnormal growth, were collected from seven sites in Baghdad governorate. These sites are the University of Baghdad, Al-Jamaa and Al-Ghazaliya neighborhood, Agricultural Research Department, Plant Protection Department, Department of Examination and Certification of Seed, and Abu Gharib district - Shiha, where these samples included the entire plant and the soil surrounding the root area. However, each sample was placed inside a polyethylene bag with the name of the area and the date of collection written on it and kept in the refrigerator for isolation procedure immediately after collecting the sample or on the next day.

2.2 Weed seed collection

The seeds of the weed plants under study (Wild Radish, wild barley) were collected from well-grown plants that do not show any disease symptoms from seven sites in Baghdad governorate that mentioned previously for the period from April to May 2019 and kept inside paper bags to prevent rotting, for use in subsequent experiments.

2.3. Seed germination test (weeds and wheat)

The germination ability and vigor of Wheat seed, var. IPA-99 was tested by Dr. Bashir from the Department of Crops- University of Baghdad and Ammar Amjad/ Department of Plant Protection. Then, the wheat seed was sterilized according to the following procedure, washing the seeds with running tap water, washing the seeds for 5 minutes in alcohol (70%), washing the seeds 3 times with sterile distilled water, soaking the seeds with diluted bleach plus 3 drops of Tween 20 for five minutes. Subsequently, the seeds were washed three times with sterile distilled water and placed on filter papers to dry, then planted in Petri dishes containing PDA culture medium and other dishes containing filter paper moistened with a little sterile distilled water. Similarly, the germination ability of both Wild Radish and Wild Barley seeds was also tested after being sterilized in the same manner as wheat and planted in Petri dishes containing PDA culture medium and other dishes containing a filter paper. Finally, few drops of sterile distilled water were added for all dishes, then they were incubated in the incubator at a temperature of 25 ± 2 °C.

2.4. Isolation and identification of *Pseudomonas* spp.

The isolation process of bacteria from soil samples that were collected previously was carried out by dilution method, where nine ml of sterilized distilled water was added for one g of the collected soil sample separately to make an aqueous suspension, and it was placed in the shaker. A series of decimal dilutions were prepared, 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and slightly shaken. Then, 0.1 ml of each dilution was transferred by syringe and pipette and placed in Petri dishes containing solid King B medium (KB) and distributed by a

sterile glass diffuser, as the notable information from the area, date, and dilution number was recorded on each dish. The samples were incubated at a temperature of 26 ± 1 °C for 24-48 hours (20, 21), where the bacteria were identified by re-culturing them on *Pseudomonas* agar medium. The isolates were preserved and maintained by growing them on Nutrient Broth (NB) medium at a temperature of 28 ± 1 °C for 48 hours, then 15% glycerin was added to them and kept in the freezer for permanent preservation. On the other hand, king B was used for temporary preservation, and the bacterial isolates were renewed after each month (22, 23, 24, 25) as follows:

2.4.1 Cultural characteristics

This stage included the observation of testing the bacterial colonies developing on the NA culture medium, which included the growth of bacterial isolates and the specifications of their colonies on the dish, the colonies shape, color, and edge, and their ability to secrete sticky substances, luster, and smell (26).

2.4.2. Molecular identification of bacteria

The Molecular identification of *Pseudomonas* sp. showed antagonistic activity against the growth of Wild Radish and Wild Barley seeds (p1) within the test depending on the 16SrRNA gene in the Polymerase chain reaction (PCR). Then, the DNA of two bacterial isolates was extracted according to the attached method with the ABIOpure extraction kit, which is supplied by the American company ABIOpure using the non-specialized universal primers 27F and 149R. Besides, the primers were prepared and supplied by Korean company Macrogen using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CTACGRRTACCTTGTTACGAC-3')(27).

2.5. Efficiency test of *Pseudomonas* spp. Isolates in inhibiting the germination of weed seeds (Wild Radish and Wild Barley) and their effect on the germination of wheat seeds

This experiment was conducted to test 12 bacterial isolates obtained from the isolated process from the area around the roots on the PDA culture media according to the method of (28). Thus, 1 ml of the bacterial isolate's suspension was added separately, which was grown on the NP liquid culture medium in a petri dish, contains the sterile PDA culture medium, then the dish was moved to distribute the bacterial inoculum homogeneously. After the medium solidification, the seeds of Wild Radish and Wild Barley were sown separately (after sterilizing them superficially with sodium hypochlorite solution for one minute) in a circular motion near the edge of the dish, with 20 seeds per dish. Besides, the treatments were repeated in four dishes for each isolate with a comparison treatment without bacteria (medium only). The dishes were incubated at $25 \pm$ °C. The results were obtained after seven days of planting and the percentage of germination was calculated.

2.6. Efficiency test of the filtrates of *Pseudomonas fluorescens* isolates in inhibiting the germination of Wild Radish and Wild Barley seeds and its effect on the germination of wheat seeds

The experiment was conducted to test the efficiency of the filters of *Pseudomonas fluorescens* isolates in inhibiting the germination of weed seeds in vitro. A liquid medium was prepared from NB medium, where 100 ml of the medium was placed in a 250 ml flask and sterilized with an autoclave (121 °C and a pressure of 1.5 kg/cm² for 15 minutes). The medium was inoculated with 1 ml of the bacterial isolate's suspension separately, which was grown on the NB culture medium at one day old. Conversely, the flasks were incubated at 25 ± 2 °C for 14 days. During this period, the flasks were shaken, then the medium was filtered by passing through two layers of medical gauze. The filtrate was collected

and sterilized by passing through a Millipore filter with a pore diameter of 0.45 μm , as the filtrate was collected in sterile flasks for each bacterial isolate separately. Finally, the filtrate was added by three ml of Petri dishes with a diameter of 9 cm container on filter paper type Whatman No.1 Coupled with, a superficially sterilized Wild Radish and Wild Barley seeds were sown on a circular motion near the edge of these dishes with 20 seeds for each one. The treatments were repeated with four dishes for each isolate with a comparison treatment (Bacterial-free media filtrate was added). The dishes were incubated at $25 \pm 2^\circ\text{C}$ and the inhibition percentage was calculated.

$$\text{Percentage of Inhibition} = \frac{\text{germinating seed number} - \text{sown seed number}}{\text{germinating seed number}} \times 100$$

3. Results

3.1. Isolation and identification of *Pseudomonas* sp.

The results of isolation and identification of the collated soil samples from seven sites (University of Baghdad, Agricultural Research Department, Plant Protection Department, Department of Examination and Certification of Seed, and Abu Gharib district – Shiha), showed that six isolates have been obtained, as these isolates were given the symbols shown in Table 1. The results also showed that *Pseudomonas* sp. was isolated from most of the samples brought to the laboratory, as these results indicate that this genus is spread around the weed roots with abnormal growth (stunting and yellowing). Moreover, the results of the growth of the different isolates on King B medium showed the growth of 6 bacterial isolates, whose agricultural characteristics were characterized as having circular colonies, smooth surfaces, regular edges, and a distinctive odor. These colonies showed a yellowish-green color in the center and their ability to move when stabbed in the semi-hard plant media, as it showed clear fog around the stabbing line. The results of the microscopic examination of these bacterial colonies also showed that their cells are short rods of Gram-negative cells and were sometimes found single or double. These results presented a good agreement with what was stated in (24). The results showed a widespread of *Pseudomonas* bacteria in local soils, in addition to the wide variation of weed plants, which agrees with (29) findings.

3.1. Molecular identification of *Pseudomonas fluorescens* isolates

The results of the PCR showed the ability of the primers 27F and 149R to identify two isolates of *Pseudomonas* sp PR4 and PR6, as they were able to the DNA pieces replication of 567 and 555 bp (base pair) respectively as shown in Figure 1. The two isolates were also recorded in the GenBank and the Accession No. was given for the two isolates (MW740144 and MW740145), respectively, which were obtained from the soil around the roots of weed plants.

Table 1. Isolates of *Pseudomonas* sp. and sites of isolation and sample type.

Isolate Symbol	Isolation site	Sample type
RP1	Baghdad/Al-Jadiriya quarter /University of Baghdad	from the soil
RP2	Baghdad / Abu Ghraib / Agricultural Research Department	from the soil
RP3	Baghdad/Abu Ghraib/ Department of Plant Protection	from the soil
RP4	Baghdad / Abu Ghraib / Department of Examination and Certification of Seed	from the soil
RP5	Baghdad/ Shiha	from the soil
RP6	Baghdad/Shiha	from the soil

The results of nucleotide sequencing analysis of the two isolate sequences that were replicated (PR4 and PR6) confirmed that they are isolates of *Pseudomonas fluorescens*. They showed 99% matching with the genetic region located between the 18S ribosomal RNA gene and 28 ribosomal when compared with the equivalent nucleotide sequences retrieved from GenBank from America and China (MW740144 and MW740145) respectively. This is the first deposit of Iraqi isolates of bacteria with the ability to inhibit weed seed germination in the GenBank.

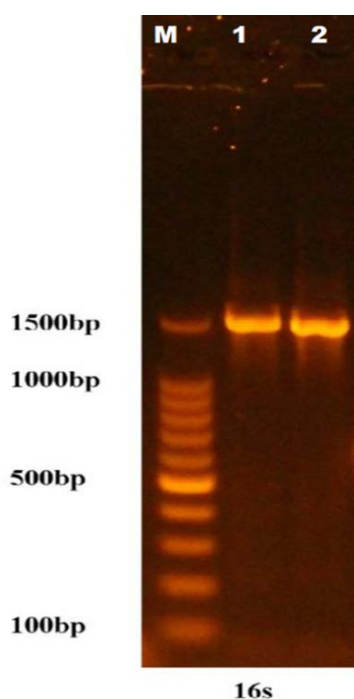


Figure 1. Electrophoresis products on agarose gel for DNA segments with molecular weight 567 and 555 bp. The numbers on the right of the figure represent the number of base pairs for the marker used. Besides, the numbers 1 and 2 represent isolates of *Pseudomonas fluorescens* (PR4 and PR6)

Figure (2) also included the Neighbor-joining phylogenetic tree that was constructed from the molecular nucleotide sequences of the two isolates (PR4 and PR6) with their counterparts from America and China (MW740144 and MW740145), respectively, Combined with, the retrieved from the GenBank of *Pseudomonas fluorescens* bacteria in one group separately from the *P. japonica* that retrieved from the GenBank that were included with the nucleotide analyzes for comparison (Outgroup) with a probability value of 50%. The Iraqi isolates showed clear genetic differences between them, as well as differences from the isolates retrieved be due to geographical variations in their source as well as differences between them in their inhibiting ability to germinate weed seeds.

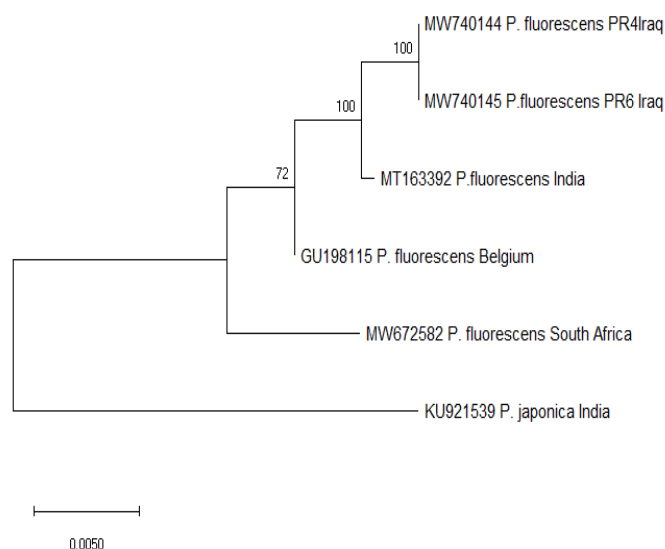


Figure 2. The genetic relationship of *Pseudomonas fluorescens* isolates. The Neighbor-joining phylogenetic tree and then its construction from the nucleotide sequences of the regions located between the 18S ribosomal RNA gene and 28 ribosomal shows the relationship between the Iraqi isolates and their counterparts retrieved from the NDBI data base.

3.2. Efficiency test of *Pseudomonas* sp. in inhibiting the germination of weed seeds (Wild Radish and Wild Barley) and its effect on the germination of wheat seeds

The results of testing the efficiency of six isolates of the genus *Pseudomonas* sp. showed that all bacterial isolates were inhibiting the germination of both Wild Radish and Wild Barley seeds as shown in Table (2). Besides that, they recorded differences in the percentages of inhibition with significant differences. The isolate PR6 was superior, as it recorded the highest percentage of inhibition of wild Radish and wild Barley seeds germination by (92.5, and 96.25%), respectively, with significant differences from the control treatment (without bacteria). Also, this isolate did not affect the germination percentage of wheat seeds, which had a germination percentage of (97.5%) compared to the comparison treatment, which had a germination percentage of (100%). Whilst the isolate PR4 scored 82.5 and 90.00% seeds germination inhibition in wild Radish and

Barley, respectively, with a significant difference from the comparison treatment. PR4 isolate scored no significant effect on the germination of wheat seeds, as its germination percentage was (92.5%) compared to the control treatment. The inhibition percentage for the rest of the isolates for seed germination of each wild radish ranged between (8.75-33.75%) and the inhibition percentage for wild barley ranged between (38.75-10.00%).

Table 2. Efficiency of *Pseudomonas* spp. isolates. in inhibiting the germination of wild radish and wild barley and its effect on the wheat crop.

Isolate	Percentage of inhibition %		Germination percentage of wheat %
	Wild radish	Wild barley	
PR1	17.5	23.75	90.00
PR2	8.75	10.00	87.5
PR3	30.00	38.75	66.25
PR4	82.50	90.00	92.50
PR5	33.75	28.75	88.75
PR6	92.50	96.25	97.50
Comparison	0.00	0.00	100.0
LSD	7.610	7.694	8.259

3.3. Efficiency test of the filtrates of *Pseudomonas* sp. isolates in inhibiting the germination of weed seeds (wild radish and wild barley) and its effect on the germination of wheat seeds

The results of the filtrate efficiency test for 6 isolates of the genus *Pseudomonas* sp. in Table (3) showed that the isolate's filtrate was inhibiting the germination of seeds of both wild radish and wild barley, and it recorded significant differences in the percentages of inhibition between the different isolates. The isolate PR6 filtrate was superior to the rest of the filtrate, as the highest percentage of inhibition was recorded for wild radish and wild barley seeds germination, which amounted to (90.00, and 93.75%), respectively, with significant differences compared to the comparison treatment (without filtrates). It also did not significantly affect the germination of wheat seeds, as its germination percentage reached (96.25 %) compared to the control treatment, which amounted to (100%). This followed by an isolate PR4 filter with a percentage of inhibition of the wild radish and wild barley germination (85.00, 88.75) %.

Table (3) The isolates efficiency of the *Pseudomonas* spp filtrate in inhibiting the germination of wild radish and wild barley seeds and their effect on wheat crop

Isolate	Percentage of inhibition %		Germination percentage of wheat %
	Wild radish	Wild barley	
PR1	13.75	26.25	86.25
PR2	6.25	5.00	87.50
PR3	33.75	40.00	65.00
PR4	85.00	88.75	87.50
PR5	25.00	27.5.0	87.50
PR6	90.00	93.75	96.25
Comparison	0.00	0.00	100.0
LSD	8.220	6.663	7.396

As well as, there is no significant difference in the percentage of inhibition between the two treatments (RP6 and RP4) and the significance has differed between it and the comparison treatment. The percentages of inhibition of wild radish and barley seed germination for the rest of the filtrates for bacterial isolate ranged from (6.25 - 33.75%) and (5.00 - 40.00%), respectively.

4. Discussion

In general, this study shows great diversity and variation in the ability of *Pseudomonas* sp. in inhibiting the germination of wild radish and barley seeds. These findings are consistent with (30) where they did a number of studies on *P. fluorescens* and found that it inhibits the growth of some weed types or that it inhibits seed germination. Similarly, (31) indicated the addition of *P. fluorescens* to the soil led to inhibiting the growth of different types of weeds including *Bromus tectorum* L, *B. japonicum*, and *Aegilops cylindrical*. (32) also pointed out that there are species of bacteria used in biological control, among the most important of which are *Xanthomonas campestris* and *P. fluorescens*. Furthermore, the biological control of weed using bacteria has advantages over the use of fungi, including that they are fast-growing and their reproduction requirements are simple, in addition to that it is easy to make genetic modifications through genetic mutations or gene transfer. However, that bacteria are among the microorganisms that have been revealed to have potential as biological control agents in weed control (13). Besides, *P. fluorescens* inhibits the

growth of some types of weeds, where (33) found that strain WH6 of *P. fluorescens* bacteria affected the germination of seeds for a wide range of weed plants (21 monocotyledons and 8 dicotyledons, but it did not affect the germination of maize (*Zea mays*) seeds. The filtrates of *P. fluorescens* could inhibit the seed germination of wild radish and barley. Similarly, (17) findings, referred to the presence of other toxic compounds in another strain *P. fluorescens* (BRG100), which was affected the growth of fox-tail weed (*Setaria viridis*). The active compound responsible for this effect was determined using Mass spectrometry and found to be L-vinylglycine 4-formlaminoxy- (10). It has been suggested that the biosynthesis of this compound begins with the amino acid homoserine (11).

5. Conclusions

Data indicated the application local isolates of *Pseudomonas fluorescens* and their filtrates enabled an ecofriendly control against weeds of wheat.

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