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Large Scale Screening of Rhizospheric Allelopathic Bacteria and Their Potential for the Biocontrol of Wheat-Associated Weeds

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Abstract: Conventional weed control practices have generated serious issues related to the environment and human health. Therefore, there is a demand for the development of alternative techniques for sustainable agriculture. The present study performed a large-scale screening of allelopathic bacteria from the rhizosphere of weeds and wheat to obtain biological weed control inoculants in the cultivation of wheat. Initially, around 400 strains of rhizobacteria were isolated from the rhizosphere of weeds as well as wheat that grows in areas of chronic weed invasions. A series of the screen was performed on these strains, including the release of phytotoxic metabolites, growth inhibition of sensitive *Escherichia coli*, growth inhibition of indicator plant of lettuce, agar bioassays on five weeds, and agar bioassay on wheat. Firstly, 22.6% (89 strains) of the total strains were cyanogenic, and among the cyanogenic strains, 21.3% (19 strains) were inhibitory to the growth of sensitive *E. coli*. Then, these 19 strains were tested using lettuce seedling bioassay to show that eight strains suppressed, nine strains promoted, and two strains remained ineffective on the growth. These 19 strains were further applied to weeds and wheat on agar bioassays. The results indicated that dry matter of broad-leaved dock, wild oat, little seed canary grass, and common lambs' quarter were reduced by eight strains (23.1–68.1%), seven strains (38.5–80.2%), eight strains (16.5–69.4%), and three strains (27.5–50.0%), respectively. Five strains suppressed the growth of wheat, nine strains increased its dry matter (12.8–47.9%), and five remained ineffective. Altogether, the strains that selectively inhibit weeds, while retaining normal growth of wheat, can offer good opportunities for the development of biological weed control in the cultivation of wheat.

Keywords: allelopathic bacteria; antimetabolites; biological control; phytotoxic metabolites; rhizobacteria; weed invasion

1. Introduction

Dramatic increases in food production have been observed in the latter half of the twentieth century owing to the use of agro-chemicals, mechanization, irrigation, high yielding varieties, and

post-harvest technology. The production of wheat in Pakistan has increased to ~25 m ton from 4.55 m ton in 1965 [1,2]. The pest attacks continue to incur losses to crop production owing to the diversity of pests and their resistance to prevailing control practices. The use of pesticides has increased from 15 to 20-fold over the last fifty years [3]. Chemical herbicides have gained importance in crop production in the face of a shortage of labor and limited application of mechanical control [4]. The mechanical control is known to contribute to soil erosion and its degradation [5]. Herbicides have led to the emergence of resistant biotypes of weeds, making the herbicide compounds useless to control these weeds [6]. Hence, the discovery of new compounds with novel modes of action is needed to replace these herbicides with more effective compounds to control such weeds. The discovery of such compounds, having herbicidal properties, has reduced over time. Further, the control of one type of weeds with herbicides has provided space to the proliferation of other weed species, which were less problematic for crop production in the past [7]. They have caused losses of biodiversity in the environment. It has deprived the ecosystems of some of their vital functions. Herbicides have aggravated the loss of biodiversity by killing the susceptible species, restricting the growth of others and the degradation of natural resources [8]. Poisoning, growth retardation, sterility, and deaths of wildlife owing to herbicide exposure have been reported by [9]. The residues of herbicides, apart from polluting the natural resources and destroying life forms, may also accumulate in the edible portions of plants, which facilitate their entry to the food chain and bodies of humans. It causes poisoning and chronic diseases in human beings, leading to deaths [10]. Human health disorders caused by herbicides include disorders of the nervous system, malformation of the embryo, loss of fertility, loss of immunity, kidney disorders, and liver disorders [11].

Farmers pay only the costs of manufacturing and marketing of herbicides, which provides economic access to farmers to adopt chemical weed control. The additional costs incurred on the treatment of human illnesses, degradation of natural resources and environment, and loss of biodiversity also need to be paid by farmers, society, or governments. Hence, the scenario of economic, environmental, and biological costs of chemical weed control pushes the researchers towards finding out safer weed control techniques. The importance of biological control has dramatically increased in the present situation. It presents a safer, inexpensive, and easier solution to the above-discussed issues of other control practices. It relies on increasing the strength, population, and activities of the organisms, resulting in growth reduction of weeds [12].

The past efforts in this area were focused on pathogens causing diseases in weeds [13] and insects feeding on weeds [14]. The success of insect biocontrol agents is limited by the existence of multiple hosts of insects in nature, which may cause the emergence of new pests of crops [15]. The pathogens of weeds used for biocontrol wait for suitable environmental conditions to cause infections and diseases in weed plants [13]. It may usually lead to delayed disease development, even after the weeds have caused economic losses of crops. Plant allelochemicals have also been investigated for biological weed control [16]. Their efficacy for weed control is reduced owing to the soil reactions, biodegradation, and mobility. It reduces their bioavailability and phytotoxicity on weeds [17]. These limitations of conventional biological weed control have discouraged researchers of this field, and the popularity of chemical weed control has increased dramatically.

The low success rate in conventional biological weed control has driven scientists to explore the characteristics of the rhizosphere inhabiting bacteria of weeds and crops for the development of novel weed biocontrol techniques. However, researchers have made efforts to explore the type of rhizobacteria, which produce substances inhibitory to the growth of weeds and are the least explored candidates for biological weed control. They release their secondary metabolites (phytotoxic in nature) in the rhizosphere, which is followed by their absorption in weeds. It results in a growth reduction of these weeds. The nature of this interaction between plants and microorganisms may be termed as plant-microbe allelopathy, and the bacteria responsible for these interactions may be called as allelopathic bacteria (AB) [18]. The discovery of host specificity in such microbial interactions with plants by [19] has opened ways for their potential application in crops for weed control. It reflects

the properties of non-inhibition or even promotion of growth of crops among these rhizobacteria [20]. Therefore, the present study was conducted to explore such bacteria from the rhizosphere of weeds and wheat growing in fields facing weed invasions chronically, characterize them for the biological weed control, and evaluate their effects on the growth of wheat and weeds species of wheat.

2. Materials and Methods

2.1. Isolation of Rhizobacteria

We collected a large pool of samples of wheat and five weeds along with earth ball across the District of Faisalabad, Punjab, Pakistan. The sampling field was selected based on chronic weed invasions over the last 5 years. The weed species sampled included field bindweed, little seed canary grass, common lambs' quarter, wild oat, and broad-leaved dock. The scientific names of these weeds are *Convolvulus arvensis*, *Phalaris minor*, *Chenopodium album*, *Avena fatua*, and *Rumex dentatus*, respectively. These samples were transferred to the laboratory in an icebox and stored at 4 °C. The rhizosphere soil of these samples was used for the isolation of rhizobacteria using the dilution plating technique. A hundred microliters of each of the serial dilutions (10^{-1}–10^{-8}) were spread on the sterilized King's B agar media in Petri plates aseptically. This media was prepared by adding 1.5-g K_2HPO_4, 10 mL glycerol, 20 gm proteose peptone, 1.5 gm $MgSO_4.7H_2O$, and 20-g agar and making up the volume of one liter with distilled water following King et al. [21]. The growth of rhizobacterial colonies was obtained after 48 h of incubation of these plates at 28 ± 1 °C. The fast-growing colonies were picked and transferred to other Petri plates containing sterilized King's B agar media. These colonies were, hence, purified after some streaking. In this way, 393 strains were purified and preserved at −20 °C in 40% glycerol.

2.2. Cyanide Production Assay on Strains of Rhizobacteria

The method given by Bakker and Schipper [22] was followed for the qualitative determination of the production of hydrogen cyanide (HCN) by the isolated strains of rhizobacteria. The pieces of filter paper to the sizes of Petri plates were made, autoclaved for sterilization, and soaked in a 1% solution of picric acid for 12 h. These soaked filter papers were dried aseptically. Glycine amended media was prepared by adding 0.35 gm K_2HPO_4, 2.5 mL glycerol, 5 gm proteose peptone, 0.35 gm $MgSO_4{\cdot}7H_2O$, 5 gm glycine, and 20-g agar and making up the volume to one liter with distilled water. It gave out quarter strength media with glycine amendment. It was autoclaved and poured in Petri plates. The fresh culture of the strains was used to make a layer on the surface of the media and placing the picric acid-soaked paper on the inner side of the Petri plate lid. The paper was fastened with the help of a 10% solution of Na_2CO_3. The plates were closed and tightened with parafilm to avoid the leakage of gas. The plates were incubated at 28 °C and periodically observed for a change in the color of filter paper. The turning of color to brown indicated the production of HCN, while the intensity of brown color indicated the level of its production (Figure 1).

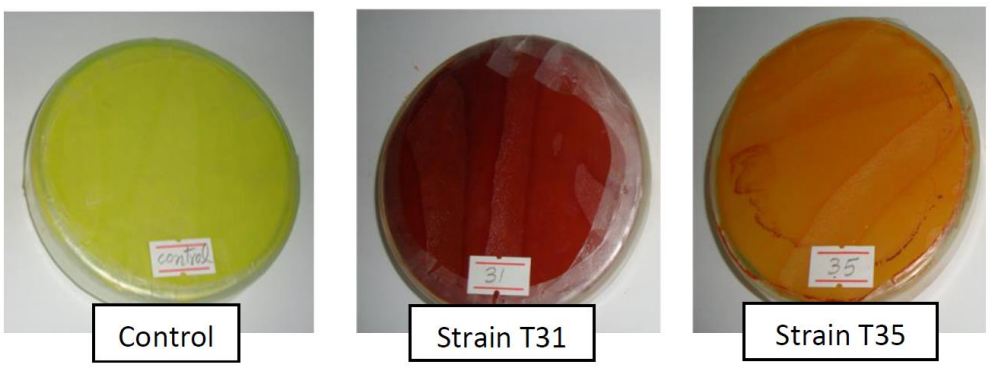

Figure 1. Pictorial view of cyanide production by rhizobacteria.

2.3. Antimetabolite Assay on E. coli

The bacterial production of toxic metabolites in extracellular spaces can be tested in a simple test based on the growth retardation of sensitive bacteria, *E. coli* [23]. All the strains (393) were tested for HCN production, while this assay was performed on only those strains that produced HCN to any level in step 1. These were 89 strains. Strain K12 of *E. coli* was cultured on LB agar media and placed in an incubator at 28 °C. After 2 days, the gentle rubbing of the surface and mixing with sterilized 0.01 M $MgSO_4$ solution formed the culture suspension of *E. coli.* The population of cells of bacteria in the suspension was maintained at 10^8 cells mL^{-1} through the measurement of optical density at 600 nm and the addition of 0.01 M $MgSO_4$ to get the value around 0.55–0.6. A layer of the harvested cell suspension was made on the Petri plates containing sterilized media (King's B). The culture of strains of cyanogenic rhizobacteria was spot inoculated at 3 points of equal distance on the plates pre-inoculated with *E. coli*. The plates were placed in an incubator at <40 °C. The production and release of toxic substances by the strains were evident from the zone of clearing around the spot of inoculation of strain. It indicated that the extracellular release of toxic compounds by the strains killed the growth of *E. coli* around its growth. The diameters of the zone of the clearing were recorded.

2.4. Antimetabolite Assay on Lettuce (Lectuca sativa L.) Seedlings

Nineteen strains restricted the growth of *E. coli* in the previous test. These strains were tested on the seedlings of lettuce as lettuce is considered sensitive to any type of phytotoxic substances and, hence, can be used as an indicator plant [24]. The fresh culture of the selected strains was prepared in Petri plates on KB media. This culture was suspended with the help of a sterilized buffer solution of $MgSO_4$ (0.01 M) by shaking gently. The suspension was collected in test tubes, and the cell population was maintained using optical density measurement at 600 nm with a value of 0.33. It established the population at 10^6 cells mL^{-1}.

The seeds of lettuce were disinfected on their surface in a parallel activity. The surface disinfection process comprised of seed dipping in ethanol for a moment, followed by the treatment with sodium hypochlorite (5%) for three minutes and complete rinsing of the seed with autoclaved water [25]. These seeds were allowed to germinate in the growth chamber.

Water agar was used as a medium for the growth of lettuce seedlings, where agar was added into the water at the rate of 1%. It was sterilized and poured in large-sized Petri plates, having a diameter of 15 cm. Seeds with good germination were picked up and transferred to the surface of these plates aseptically. Twenty germinating seeds of lettuce were placed on each plate.

Thirty microliters of the bacterial cell suspension were dispensed to each seed for inoculation. Three Petri plates were prepared for each strain in the same way. The control plates were treated with 30 µL buffer (0.01 M $MgSO_4$) per seed. The plates were placed at ambient temperature in the dark for 4 days. Then, the seedlings were removed from the plates and blotted. The measurements of masses and lengths of roots and shoots were done. The data were analyzed statistically to determine the significant differences [26].

2.5. Antimetabolite Assay on Weeds Using Presumed Allelopathic Bacteria

The strains of rhizobacteria obtained after the above-mentioned steps of the screening process were now called as presumed allelopathic bacteria. These strains were, now, used for testing on weeds. We selected four weeds of wheat for this assay i.e., wild oat, broad-leaved dock, common lambs' quarter, and little seed canary grass. These weeds cause maximum economic losses in the wheat crop in Pakistan. Nineteen strains were used to conduct this study in an experimental set up similar to the one used for bioassay on lettuce seedlings in Section 2.4. The culture of each strain was prepared in King's B broth. The culture was centrifuged to get the supernatant and form the bacterial pellets. These pellets were mixed in a sterilized buffer (0.01 M $MgSO_4$) to adjust the optical density value of 0.55 at 600 nm. It gave out the bacterial cell population at 10^8 cells mL^{-1}.

Water agar was prepared by adding 10 g of agar in 1 L distilled water and sterilizing in an autoclave at 121 °C and 15 PSI pressure for 20 min. The water agar was poured on large-sized Petri plates. It served as a medium for the growth of seedlings (Figure 2).

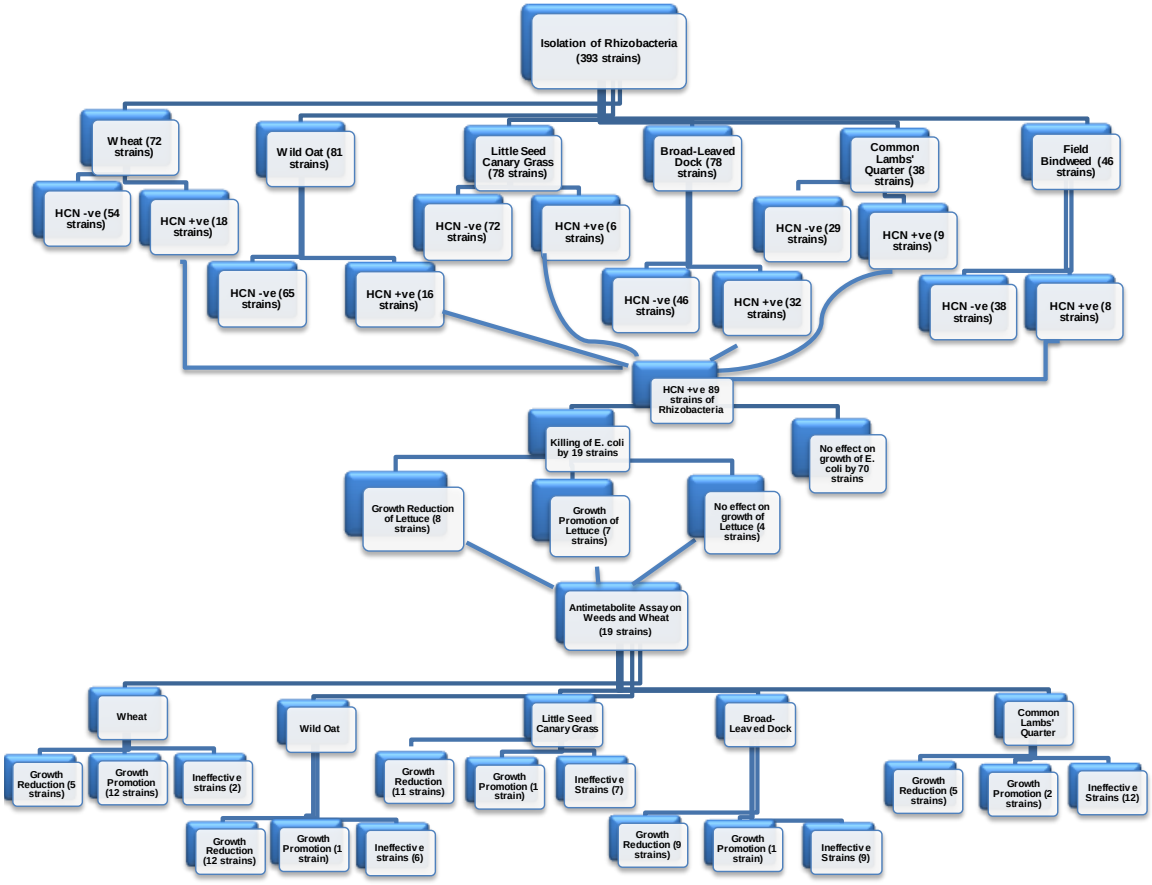

Figure 2. Flow chart of isolation and large-scale screening of allelopathic bacteria for the biocontrol of wheat-associated weeds.

The seeds of the selected weeds were surface disinfected by washing with ethanol (70%) momentarily, followed by washing with sodium hypochlorite (5%) and rinsing of seeds in plenty of sterilized water [25]. These seeds were placed in the growth chamber for germination.

Twenty germinated seeds were placed inside each prepared Petri plates aseptically. The culture suspension of each strain was applied at the rate of 30 µL per seed. For the control treatment, the sterilized buffer (0.01 M $MgSO_4$) was applied at the same rate. The plates were placed at ambient temperature in the dark. Each treatment in the experiment was replicated four times. After 7 days, the seedlings were uprooted from the water agar plates and blotted. These seedlings were measured for the lengths and weights of roots and shoots. The data were analyzed statistically to determine the significant differences following Steel et al. [26].

2.6. Antimetabolite Assay on Wheat Using Presumed Allelopathic Bacteria

The same nineteen strains were also tested for their effects on the growth of seedlings of wheat in a similar agar bioassay (Figure 3). The culture suspension of the strains was prepared following the same method as above. The large-sized Petri plates containing water agar were prepared as in previous bioassays. The surface of seeds of wheat was disinfected following Abd-Alla et al. [25]. Then, the seeds were placed for germination. The germinated seeds were placed on the already prepared Petri plates aseptically. The culture suspension of each strain was dispensed at the rate of 30 µL per seed. For the

control treatment, the sterilized buffer (0.01 M $MgSO_4$) was dispensed to each seed at the rate of 30 µL. Each treatment was replicated four times. The seedlings were uprooted after five days and blotted. The data of lengths and weights of roots and shoots were taken and analyzed statistically to determine the significant differences following Steel et al. [26]. These analyses were carried out using *Statistix 8.1* software. All the data were first subjected to analysis of variance (ANOVA) test in this software, followed by multiple comparisons of means using the linear model. The least significant difference (LSD) test was then applied to determine the significant difference among treatments at $p < 0.05$.

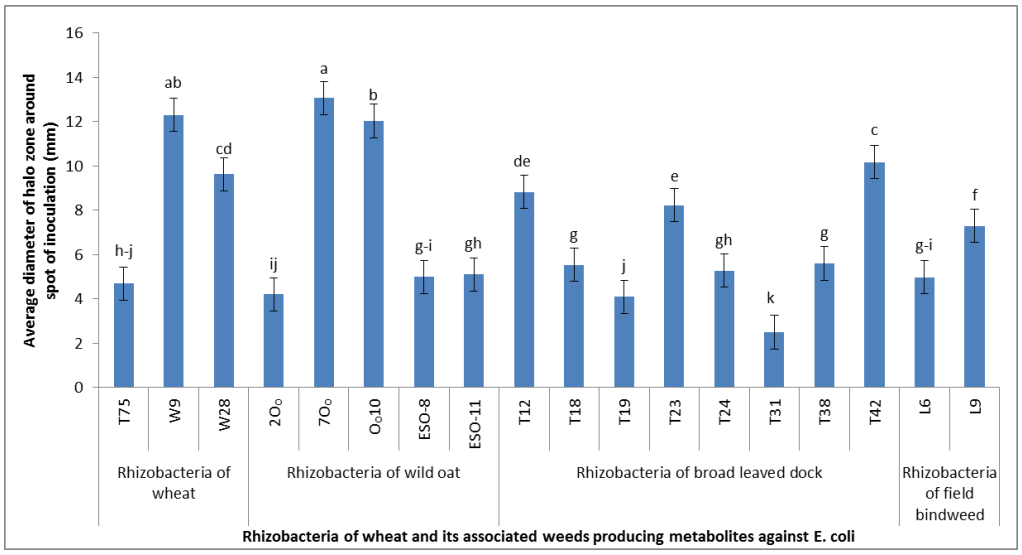

Figure 3. Cyanogenic rhizobacteria of weeds and wheat-producing metabolites against *E. coli* in the antimetabolite assay.

2.7. Cluster Analysis for the Screening of Biological Weed Control Agents

Cluster analysis was carried out for the grouping of strains applied in antimetabolite assays on weeds and wheat. The strains were categorized as non-selective biological weed control agents (the strains that reduced the growth of all the tested weeds and wheat), selective (the strains that reduced the growth of some of the tested plants and also wheat), selective (the strains that reduced the growth of some of the weeds but not wheat), and selective (the strains that reduced the growth of one more weed but promoted the growth of wheat). Five most efficient strains of allelopathic bacteria obtained from this study were identified through 16s rDNA sequencing as *Pseudomonas* strain T42 as *Pseudomonas putida*, strains L9 and $7O_0$ as *P. fluorescens*, strain $O_0$10 as *P. aeruginosa*, and strain W9 as *P. alcaligenes*.

3. Results

The present study explored the rhizosphere of wheat and five weeds of wheat in search of allelopathic bacteria for the development of biological weed control agents. The selected weeds cause huge economic losses to the production of wheat in Pakistan annually [27]. These weed species were wild oat, common lambs' quarter, little seed canary grass, broad-leaved dock, and field bindweed. The job was carried out by the isolation of a large number of strains of rhizobacteria (393) from the weeds and wheat growing in areas of high weed invasion. Multiple bioassays were conducted on these strains to evaluate if they produced some phytotoxic substances, whether the release of such substances resulted in growth suppression of weeds, and if they were selective to inhibit the growth of weeds but not crop. The screening process of rhizobacteria to find out allelopathic bacteria from the rhizosphere of weeds and wheat is shown in the form of a flow chart (Figure 2).

3.1. Isolation of Rhizobacteria

We isolated 78 strains from the rhizosphere of wild oat, 81 from the broad-leaved dock, 78 from common lambs' quarter, 46 from field bindweed, 38 from little seed canary grass, and 72 from wheat. The total number of strains was 393. Multiple screening tests were conducted on these strains to characterize weed suppressive allelopathic bacteria.

3.2. Production of HCN by Rhizobacteria

The proportion of strains producing cyanide to various levels is shown in Table 1. We got 89 strains, which could produce cyanide to any level. Among these, 33 strains produced a low amount of cyanide, 25 medium, 20 high, and 11 very high, depending upon the intensity of change of color of picrate-treated filter paper inside the Petri plates and the time taken to change the color. The proportion of cyanogenic strains in the rhizosphere of the broad-leaved dock was calculated to be 41.0%, that of wild oat was 19.8%, of little seed canary grass was 7.7%, of common lambs' quarter was 23.7%, of field bindweed was 17.4%, and that of wheat was 25.0%. However, the majority of strains (77.6%) did not produce HCN in this study. These counted to 304 in number out of 393. The pictorial view of this assay is given in (Figure 1).

Table 1. The proportion of cyanogenic rhizobacteria in the rhizosphere of wheat and its associated weeds. The cyanide production by the strains was indicated after 48, 36, 24, and 12 h of incubation for low, medium, high, and very high cyanide production activity, respectively.

Category	Rhizosphere of						Total Strains
	Wheat	Broad-Leaved Dock	Wild Oat	Little Seed Canary Grass	Field Bindweed	Common Lambs' Quarter	
Non-cyanogenic strains	54	46	65	72	38	29	304
Low cyanide activity strains	8	3	8	5	3	6	33
Medium cyanide activity strains	6	12	3	0	2	2	25
High cyanide activity strains	2	14	0	1	2	1	20
Very high cyanide activity strains	2	3	5	0	1	0	11
Total strains	72	78	81	78	46	38	393

3.3. Antimetabolite Assay on E. coli

Clearing zones were produced around the inoculation spot of some strains, while the growth of most of the strains was mixed with the growth of *E. coli*, i.e., mutualistic strains. The clearing zones indicated the killing of *E. coli*, which occurred with nineteen strains. The diameter of these clearing or halo zones indicated the level of inhibition of growth of *E. coli* (Figure 3). Strain $7O_0$ produced the maximum diameter of the halo zone, which was followed by strains W9, $O_0$10, T42, W28, T12, T23, and L9. The average diameter of zones produced by these strains was measured to be 1.3 ± 0.08, 1.23 ± 0.13, 1.21 ± 0.08, 1.01 ± 0.10, 0.96 ± 0.09, 0.88 ± 0.06, 0.82 ± 0.06, and 0.72 ± 0.07 cm, respectively. The remaining strains showed positive interaction with the growth of *E. coli*.

3.4. Antimetabolite Assay on Lettuce Seedlings

Results indicated that the strains imparted mixed effects on the growth of lettuce seedlings (Table 2). Five of the application strains significantly reduced the dry matter, root length, and shoot length of lettuce seedlings from 18.8 to 38.9%, 19.7 to 36.3%, and 17.3 to 24.3%, respectively. These strains were T18, T12, W9, W28, and $O_0$10. The strains L6 and T31 caused a significant reduction in root length only. However, the strain T38 caused a significant reduction in the length of root and shoot. There were

seven strains, which increased the dry matter, root length, and shoot length of lettuce seedlings from 15.7 to 41.5%, 16.7 to 61.4%, and 26.2 to 43.4%, respectively. These strains were T23, T42, T19, $2O_0$, T24, L9, and $7O_0$. The strains B11 and ESO-8 increased the shoot length only. The other strains remained ineffective on the growth of seedlings of lettuce.

Table 2. The effect of cyanogenic *E. coli* inhibiting rhizobacteria on lettuce seedlings in agar bioassay. Values sharing the same letter(s) in a column do not differ significantly from each other at $p < 0.05$. Values in a column indicate mean ± standard error.

Treatments	Root Length (cm)	Shoot Length (cm)	Dry Matter (mg)
Control	5.08 ± 0.14 ^{d,e}	4.01 ± 0.13 ^{f,g}	51.49 ± 0.006 ^{f,g}
T12	4.07 ± 0.16 ^{g,h}	3.19 ± 0.10 ^{i}	38.94 ± 0.006 ^{i,j}
T18	3.79 ± 0.16 ^{h,i}	3.32 ± 0.09 ^{i}	41.81 ± 0.006 ^{h,i,j}
T19	7.1 ± 0.13 ^{b}	5.39 ± 0.03 ^{b,c}	67.13 ± 0.006 ^{a,b}
T23	6.08 ± 0.12 ^{c}	5.22 ± 0.13 ^{c}	61.7 ± 0.010 ^{b,c,d}
T24	5.92 ± 0.27 ^{c}	5.07 ± 0.15 ^{c}	59.57 ± 0.007 ^{c,d,e}
T31	4.36 ± 0.20 ^{f,g}	3.68 ± 0.12 ^{g,h}	44.2 ± 0.007 ^{h,i}
T38	4.06 ± 0.16 ^{g,h}	3.37 ± 0.08 ^{h,i}	42.07 ± 0.007 ^{hij}
T42	6.35 ± 0.22 ^{c}	4.63 ± 0.15 ^{d}	58.42 ± 0.007 ^{d,e}
T75	4.8 ± 0.26 ^{e,f}	4.02 ± 0.15 ^{f}	50.7 ± 0.003 ^{f,g}
$2O_0$	6.19 ± 0.20 ^{c}	5.16 ± 0.14 ^{c}	65.26 ± 0.007 ^{b,c}
$7O_0$	8.19 ± 0.17 ^{a}	5.76 ± 0.11 ^{a}	72.19 ± 0.006 ^{a}
O_010	3.4 ± 0.14 ^{i,j}	3.18 ± 0.09 ^{i}	39.78 ± 0.006 ^{hij}
ESO-8	5.37 ± 0.20 ^{d}	4.43 ± 0.17 ^{d}	55.0 ± 0.003 ^{e,f}
ESO-11	5.03 ± 0.25 ^{d,e}	4.06 ± 0.11 ^{e,f}	52.0 ± 0.007 ^{f,g}
L6	4.48 ± 0.13 ^{f,g}	3.79 ± 0.11 ^{f,g}	45.79 ± 0.003 ^{g,h}
L9	8.01 ± 0.19 ^{a}	5.73 ± 0.05 ^{a,b}	72.88 ± 0.007 ^{a}
B11	5.26 ± 0.11 ^{d,e}	4.38 ± 0.18 ^{d,e}	54.53 ± 0.006 ^{e,f}
W9	3.34 ± 0.12 ^{i,j}	3.11 ± 0.08 ^{i}	35.99 ± 0.003 ^{j}
W28	3.231 ± 0.19 ^{j}	3.04 ± 0.14 ^{i}	38.08 ± 0.003 ^{i,j}
LSD	0.517	0.345	6.42

3.5. Antimetabolite Assay on Broad-Leaved Dock

The effects of the applied strains on the growth of the seedling of the broad-leaved dock were mixed, i.e., inhibiting, promoting, and neutral (Table 3). The dry matter, root length, and germination rate of the broad-leaved dock were significantly reduced by eight of the applied strains from 23.1 to 68.1%, 23.9 to 61.8%, and 26.7 to 64.4% than control, respectively. These strains were T42, O_010, L9, T38, $7O_0$, ESO-11, W9, and W28. The strain T19 caused a reduction in root length and germination rate only. The strain T31 caused a significant increase in root length and germination rate of the dock. The other strains remained ineffective on the growth of the seedlings of the dock.

3.6. Antimetabolite Assay on Wild Oat

Seven strains significantly reduced the dry matter, root length, and germination rate of wild oat from 38.5 to 80.2%, 19.4 to 60.2%, and 25.4 to 70.9%, respectively (Table 3). These strains were $2O_0$, ESO-8, O_010, T42, W28, W9, and $7O_0$. The strains T18, T12, ESO-11, and T75 significantly inhibited the germination rate from 14.5 to 25.4% but no other parameters. The strain T24 only reduced the root length of wild oat. The root length and germination rate of the weed were significantly increased by strain T19 up to 13.3 and 14.5%, respectively. The other strains remained ineffective on the growth of the seedlings of wild oat.

3.7. Antimetabolite Assay on Little Seed Canary Grass

Eight of the nineteen applied strains caused a significant reduction in dry matter, root length, and germination rate of little seed canary grass from 16.5 to 69.4%, 24.2 to 63.6%, and 20 to 52.7%,

respectively (Table 4). These eight strains were T75, $7O_0$, T42, ESO-11, $O_0$10, W9, L9, and W28. The strains T18 and T12 reduced only the root length (10.5–20%) and germination rate (18.2–25.4%). The strain $2O_0$ significantly reduced the dry matter (21.2%) and root length (10.8%) of the weed. However, the strain T19 significantly increased the dry matter (23.5%) and root length (10.4%) of the weed. Other strains remained ineffective on the growth of the seedlings of this weed. The pictorial view of the assay is available in (Figure 4).

Table 3. The effect of presumed allelopathic bacteria on the germination and seedling growth of broad-leaved dock and wild oat in agar bioassay. Values sharing the same letter(s) in a column do not differ significantly from each other at $p < 0.05$. Values in a column indicate mean ± standard error.

Treatment	Broad-Leaved Dock			Wild Oat		
	Germination Rate (%)	Root Length (cm)	Dry Matter (g)	Germination Rate (%)	Root Length (cm)	Dry Matter (g)
Control	75.0 ± 0.58 ^{b,c}	3.52 ± 0.13 ^{b}	0.307 ± 0.014 ^{a,b,c}	73.3 ± 0.33 ^{b,c}	6.0 ± 0.16 ^{b,c,d}	0.32 ± 0.03 ^{b,c,d}
T12	73.4 ± 0.88 ^{b,c}	3.48 ± 0.12 ^{b}	0.29 ± 0.035 ^{a,b,c,d}	62.7 ± 0.88 ^{d,e,f}	5.6 ± 0.17 ^{c,d,e}	0.29 ± 0.02 ^{b,c,d}
T18	80.0 ± 1.00 ^{a,b}	3.5 ± 0.10 ^{b}	0.303 ± 0.026 ^{a,b,c}	58.7 ± 0.67 ^{f,g}	5.52 ± 0.24 ^{d,e}	0.28 ± 0.02 ^{c,d}
T19	63.4 ± 0.67 ^{d,e}	2.98 ± 0.18 ^{c,d}	0.247 ± 0.013 ^{c,d,e}	84.0 ± 0.58 ^{a}	6.79 ± 0.13 ^{a}	0.41 ± 0.03 ^{a}
T23	68.3 ± 0.33 ^{c,d}	3.33 ± 0.11 ^{b,c}	0.277 ± 0.018 ^{b,c,d}	66.7 ± 0.33 ^{c,d,e}	6.16 ± 0.16 ^{b}	0.30 ± 0.02 ^{b,c,d}
T24	71.6 ± 0.33 ^{b,c,d}	3.46 ± 0.11 ^{b}	0.297 ± 0.023 ^{a,b,cd}	68.0 ± 0.58 ^{c,d}	5.28 ± 0.14 ^{e,f}	0.29 ± 0.02 ^{b,c,d}
T31	86.6 ± 0.33 ^{a}	4.1 ± 0.23 ^{a}	0.353 ± 0.014 ^{a}	73.3 ± 0.33 ^{b,c}	5.75 ± 0.15 ^{b,c,d,e}	0.32 ± 0.02 ^{b,c,d}
T38	55.0 ± 0.58 ^{e,f}	2.68 ± 0.28 ^{d,e}	0.233 ± 0.017 ^{d,e,f}	77.3 ± 0.33 ^{a,b}	5.96 ± 0.08 ^{b,c,d}	0.33 ± 0.02 ^{b,c}
T42	33.3 ± 0.67 ^{i,j}	1.73 ± 0.29 ^{h,i}	0.13 ± 0.020 ^{h,i}	32.0 ± 0.58 ^{k}	2.74 ± 0.18 ^{j}	0.11 ± 0.01 ^{h,i}
T75	70.0 ± 1.00 ^{c,d}	3.33 ± 0.06 ^{b,c}	0.28 ± 0.036 ^{b,c,d}	60.0 ± 1.00 ^{e,f,g}	5.88 ± 0.33 ^{b,c,d}	0.28 ± 0.02 ^{b,c,d}
$2O_0$	73.4 ± 0.33 ^{b,c}	3.61 ± 0.14 ^{b}	0.303 ± 0.022 ^{a,b,c}	54.7 ± 0.88 ^{g,h}	4.55 ± 0.18 ^{g}	0.20 ± 0.01 ^{e,f}
$7O_0$	41.7 ± 0.33 ^{h,i}	1.96 ± 0.06 ^{g,h}	0.17 ± 0.023 ^{f,g,h}	49.3 ± 0.67 ^{h,i}	3.77 ± 0.22 ^{h,i}	0.16 ± 0.003 ^{f,g,h}
$O_0$10	51.6 ± 0.33 ^{f,g}	2.17 ± 0.10 ^{f,g}	0.203 ± 0.027 ^{e,f,g}	45.3 ± 0.67 ^{i,j}	3.6 ± 0.19 ^{i}	0.15 ± 0.03 ^{f,g,h}
ESO-8	73.4 ± 0.67 ^{b,c}	3.41 ± 0.21 ^{b}	0.29 ± 0.026 ^{a,c,d}	60.0 ± 0.58 ^{e,f,g}	4.83 ± 0.18 ^{f,g}	0.20 ± 0.01 ^{f,g}
ESO-11	48.4 ± 1.20 ^{f,g,h}	2.53 ± 0.06 ^{e,f}	0.203 ± 0.018 ^{e,f,g}	54.7 ± 0.88 ^{g,h}	5.56 ± 0.29 ^{d,e}	0.26 ± 0.02 ^{d,e}
L6	56.6 ± 0.67 ^{e,f}	2.77 ± 0.08 ^{d,e}	0.24 ± 0.020 ^{c,d,e}	82.7 ± 0.33 ^{a}	6.13 ± 0.17 ^{b,c}	0.35 0.03 ^{a,b}
L9	41.6 ± 0.67 ^{h,i}	1.88 ± 0.10 ^{g,h}	0.157 ± 0.022 ^{g,h,i}	21.3 ± 0.33 ^{l}	2.39 ± 0.23 ^{j}	0.63 ± 0.01 ^{i}
B11	76.6 ± 0.67 ^{b,c}	3.65 ± 0.11 ^{b}	0.317 ± 0.033 ^{a,b}	70.7 ± 0.33 ^{b,c}	6.0 ± 0.19 $^{b–d}$	0.32 ± 0.02 ^{b,c,d}
W9	26.6 ± 0.67 ^{j}	1.34 ± 0.12 ^{i}	0.097 ± 0.018 ^{i}	41.3 ± 0.33 ^{j}	3.42 ± 0.13 ^{i}	0.13 ± 0.03 ^{g,h}
W28	43.4 ± 0.88 ^{g,h}	1.41 ± 0.08 ^{i}	0.14 ± 0.029 ^{g,h,i}	44.0 ± 1.16 ^{i,j}	4.3 ± 0.13 ^{g,h}	0.17 ± 0.02 ^{f,g,h}
LSD	9.8205	0.429	0.068	7.32	0.545	0.0642

Table 4. The effect of presumed allelopathic bacteria on the germination and seedling growth of little seed canary grass and common lambs' quarter. Values sharing the same letter(s) in a column do not differ significantly from each other at $p < 0.05$. Values in a column indicate mean ± standard error.

Treatments	Little Seed Canary Grass			Common Lambs' Quarter		
	Germination Rate (%)	Root Length (cm)	Dry Matter (g)	Germination Rate (%)	Root Length (cm)	Dry Matter (g)
Control	73.3 ± 1.20 ^{a,b}	4.59 ± 0.22 ^{b,c}	0.283 ± 0.026 ^{b,c}	63.3 ± 1.00 ^{c,d,e}	2.87 ± 0.19 ^{b,c,d,e}	0.27 ± 0.01 ^{a,b,c}
T12	54.7 ± 1.45 ^{d,e,f,g}	4.11 ± 0.06 ^{d,e}	0.287 ± 0.024 ^{b,c}	61.0 ± 0.67 ^{c,d,e}	2.57 ± 0.12 ^{d,e,f,g}	0.25 ± 0.02 ^{b,c,d}
T18	60.0 ± 1.53 ^{c,d,e,f}	3.68 ± 0.08 ^{e,f}	0.267 ± 0.026 ^{b,c,d}	61.5 ± 0.88 ^{c,d,e}	2.29 ± 0.11 ^{f,g,h}	0.24 ± 0.01 ^{b,c,d}
T19	81.3 ± 0.67 ^{a}	5.07 ± 0.11 ^{a}	0.35 ± 0.021 ^{a}	63.2 ± 1.00 ^{c,d,e}	2.50 ± 0.17 ^{e,f,g,h}	0.27 ± 0.01 ^{a,b,c}
T23	66.7 ± 0.33 ^{b,c}	4.64 ± 0.15 ^{a,b}	0.26 ± 0.015 ^{b,c,d}	58.7 ± 1.45 ^{c,d,e}	2.73 ± 0.11 ^{c,d,e,f}	0.25 ± 0.02 ^{b,c,d}
T24	68.0 ± 0 ^{b,c}	4.28 ± 0.16 ^{b,c,d}	0.237 ± 0.013 ^{c,d,e}	66.7 ± 1.00 ^{b,c}	3.14 ± 0.22 ^{a,b,c}	0.29 ± 0.01 ^{a,b}
T31	74.7 ± 0.33 ^{a,b}	4.32 ± 0.08 ^{b,c,d}	0.287 ± 0.014 ^{b,c}	57.7 ± 0.33 ^{d,e,f}	2.9 ± 0.22 ^{b,c,d,e}	0.28 ± 0.06 ^{a,b}
T38	74.7 ± 0.67 ^{a,b}	4.58 ± 0.11 ^{b,c}	0.277 ± 0.024 ^{b,c}	57.2 ± 0.33 ^{d,e,f}	2.61 ± 0.28 ^{d,e,f}	0.24 ± 0.03 ^{b,c,d}
T42	54.7 ± 0.88 ^{d,e,f,g}	2.89 ± 0.10 ^{g,h}	0.153 ± 0.017 ^{g,h,i}	65.7 ± 0.67 ^{b,c,d}	3.04 ± 0.19 ^{a,b,c,d}	0.24 ± 0.04 ^{b,c,d}
T75	53.3 ± 1.67 ^{e,f,g,h}	2.34 ± 0.09 ^{i}	0.175 ± 0.005 ^{f,g,h}	47.7 ± 0.67 ^{g}	2.04 ± 0.17 ^{h,i}	0.19 ± 0.02 ^{d,e,f}
$2O_0$	64.0 ± 1.00 ^{b,c,d,e}	4.1 ± 0.24 ^{d,e}	0.223 ± 0.007 ^{d,e,f}	75.6 ± 0.33 ^{a}	3.51 ± 0.09 ^{a}	0.32 ± 0.01 ^{a}
$7O_0$	42.7 ± 0.67 ^{h,i}	2.37 ± 0.18 ^{i}	0.100 ± 0.006 ^{j}	55.7 ± 0.33 ^{e,f,g}	2.90 ± 0.17 ^{b,c,d,e}	0.24 ± 0.01 ^{b,c,d,e}
$O_0$10	34.7 ± 0.67 ^{i}	1.8 ± 0.19 ^{j}	0.087 ± 0.019 ^{j}	63.3 ± 0.58 ^{c,d,e}	2.93 ± 0.11 ^{b,c,d,e}	0.24 ± 0.02 ^{b,c,d}
ESO-8	65.3 ± 0.88 ^{b,c,d}	4.16 ± 0.21 ^{c,d}	0.237 ± 0.012 ^{c,d,e}	64.3 ± 0.67 ^{b,c,d}	2.93 ± 0.27 ^{b,c,d,e}	0.28 ± 0.02 ^{a,b,c}
ESO-11	48.0 ± 1.53 ^{g,h}	3.48 ± 0.08 ^{f}	0.237 ± 0.022 ^{c,d,e}	49.0 ± 0.67 ^{g}	1.69 ± 0.14 ^{i,j}	0.17 ± 0.02 ^{e,f}
L6	80.0 ± 0.58 ^{a}	4.57 ± 0.11 ^{b,c}	0.293 ± 0.013 ^{b}	72.3 ± 0.88 ^{a,b}	3.32 ± 0.25 ^{a,b}	0.30 ± 0.01 ^{a,b}
L9	49.3 ± 0.67 ^{f,g,h}	2.54 ± 0.20 ^{h,i}	0.123 ± 0.007 ^{i,j}	63.3 ± 1.53 ^{c,d,e}	2.69 ± 0.12 ^{c,d,e,f}	0.27 ± 0.04 ^{a,b,c}
B11	74.7 ± 0.67 ^{a,b}	4.52 ± 0.11 ^{b,c,d}	0.257 ± 0.012 ^{b,c,d}	49.0 ± 0.88 ^{g}	2.09 ± 0.11 ^{g,h,i}	0.21 ± 0.01 ^{c,d,e}
W9	58.7 ± 0.33 ^{c,d,e,f,g}	3.26 ± 0.27 ^{f,g}	0.197 ± 0.032 ^{e,f,g}	59.0 ± 0.67 ^{c,d,e}	2.98 ± 0.29 ^{b,c,d,e}	0.26 ± 0.03 ^{a,b,c,d}
W28	49.3 ± 1.77 ^{f,g,h}	1.67 ± 0.09 ^{j}	0.137 ± 0.003 ^{h,i,j}	50.0 ± 1.16 ^{f,g}	1.29 ± 0.13 ^{j}	0.13 ± 0.02 ^{f}
LSD	11.432	0.445	0.0507	8.127	0.524	0.073

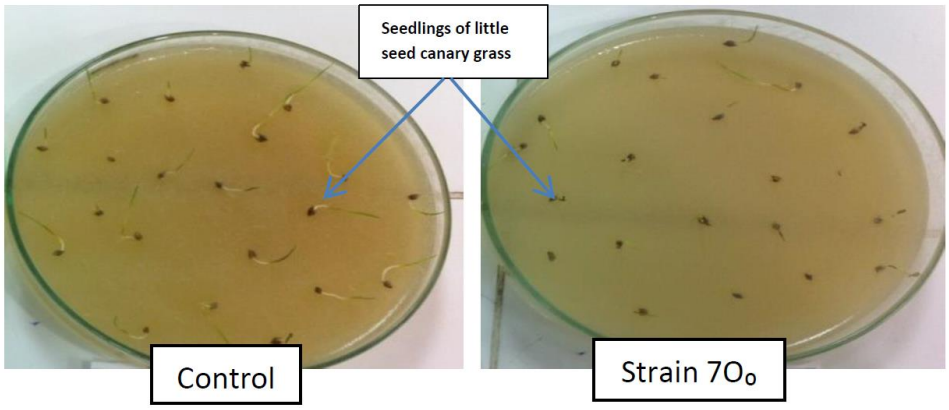

Figure 4. The pictorial view of seedlings of little seed canary grass growing on water agar in agar bioassay.

3.8. Antimetabolite Assay on Common Lambs' Quarter

The present study reported a decrease in dry matter, root length, and germination rate of common lambs' quarter by three of the applied strains from 27.5 to 50.0%, 29.0 to 55.0%, and 21.0 to 24.6%, respectively (Table 4). These strains were W28, ESO-11, and T75. The strain B11 caused a reduction in root length (27.3%) and germination rate (22.8%) only. The strain T18 caused a reduction in root length only, which was 20.3% lesser than the control. However, a significant increase in root length (13.0%) and germination rate (19.3%) was observed with the inoculation of strain $2O_0$. The strain L6 increased the germination rate of the weed by 14%. The other strains remained ineffective on the growth of the seedlings of this weed.

3.9. Antimetabolite Assay on Wheat

There were three strains in the whole lot, which significantly reduced the dry matter, shoot length, root length, and germination rate of wheat from 23.4 to 34%, 21.0 to 38.5%, 27.2 to 52.8%, and 8.3 to 10.4%, respectively (Figure 5, Table 5). These three strains were ESO-11, W28, and T18. Two strains (T75 and T12) reduced the dry matter (23.4 and 26.6%), root length (24.8 and 50.1%), and shoot length (18.9 and 35.5%) of the crop. However, there were six strains, which significantly increased the dry matter, shoot length, root length, and germination rate of wheat from 24.5 to 47.9%, 14.6 to 29.7%, 19.4 to 37.7%, and 12.5 to 18.8%, respectively. These strains were T23, $7O_0$, $2O_0$, L9, T24, and T19. The strains L6, $O_0$10, and B11 caused an increment in dry matter of the crop up to 13.8, 12.8, and 27.7% than control, respectively. The strains T38 and T31 caused a significant increase in shoot length of the crop up to 18.9 and 18.7% than control, respectively. The strain T42, however, increased the germination rate of the crop up to 8.3%. The other strains remained ineffective on the growth of the seedlings of wheat.

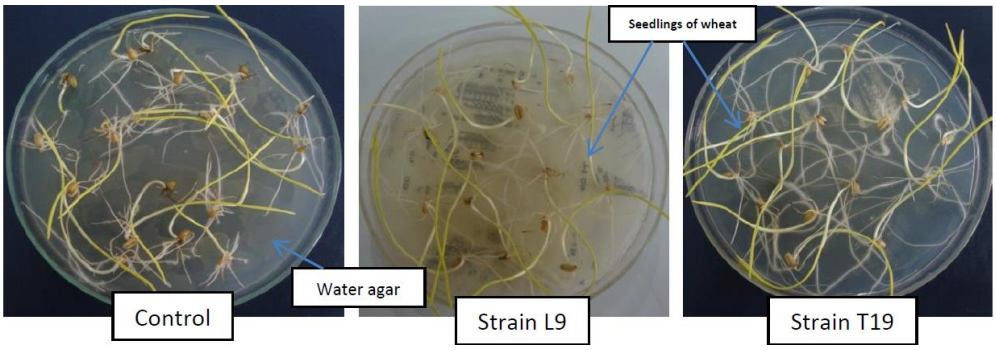

Figure 5. The pictorial view of seedlings of wheat growing on water agar in agar bioassay.

Table 5. The effect of presumed allelopathic bacteria on the germination and seedling growth of wheat. Values sharing the same letter(s) in a column do not differ significantly from each other at $p < 0.05$. Values in a column indicate mean ± standard error.

Treatments	Germination Rate (%)	Root Length (cm)	Shoot Length (cm)	Dry Matter (g)
Control	80.0 ± 0.58 ^{d,e}	6.60 ± 0.50 ^{c,d,e}	8.58 ± 0.22 ^{c,d}	0.313 ± 0.018 ^{f,g}
T12	75.0 ± 0.58 ^{e,f}	4.97 ± 0.08 ^{f}	6.96 ± 0.27 ^{e}	0.233 ± 0.003 ^{h}
T18	73.35 ± 0.33 ^{f}	4.81 ± 0.10 ^{f}	6.78 ± 0.16 ^{e}	0.240 ± 0.015 ^{h}
T19	93.4 ± 0.33 ^{a}	8.22 ± 0.15 ^{b}	9.83 ± 0.37 ^{b}	0.407 ± 0.012 ^{c}
T23	90.0 ± 0.58 ^{a,b}	8.13 ± 0.14 ^{b}	10.13 ± 0.34 ^{b}	0.390 ± 0.006 ^{c,d}
T24	90.0 ± 0 ^{a,b}	7.92 ± 0.13 ^{b}	10.05 ± 0.21 ^{b}	0.400 ± 0.015 ^{c}
T31	80.0 ± 0.58 ^{d,e}	6.47 ± 0.23 ^{d,e}	10.19 ± 0.20 ^{b}	0.300 ± 0.020 ^{g}
T38	80.0 ± 0.58 ^{d,e}	6.67 ± 0.27 ^{c,d,e}	10.21 ± 0.17 ^{b}	0.310 ± 0.015 ^{f,g}
T42	86.5 ± 0.33 ^{b,c}	7.15 ± 0.25 ^{c}	9.097 ± 0.38 ^{c}	0.343 ± 0.012 ^{e,f}
T75	75.0 ± 0 ^{e,f}	3.3 ± 0.17 ^{g}	5.53 ± 0.10 ^{f}	0.230 ± 0.012 ^{h}
$2O_0$	93.4 ± 0.33 ^{a}	7.89 ± 0.26 ^{b}	10.03 ± 0.18 ^{b}	0.413 ± 0.003 ^{b,c}
$7O_0$	93.4 ± 0.33 ^{a}	9.09 ± 0.21 ^{a}	11.13 ± 0.25 ^{a}	0.450 ± 0.010 ^{a,b}
O_010	85 ± 0 $^{b–d}$	7.17 ± 0.15 ^{c}	8.69 ± 0.19 ^{c,d}	0.353 ± 0.003 ^{d,e}
ESO-8	81.5 ± 0.67 ^{c,d}	6.53 ± 0.17 $^{c–e}$	8.52 ± 0.16 ^{c,d}	0.347 ± 0.014 ^{e,f}
ESO-11	73.4 ± 0.67 ^{f}	3.38 ± 0.33 ^{g}	5.56 ± 0.15 ^{f}	0.220 ± 0.006 ^{h}
L6	83.4 ± 0.33 ^{c,d}	6.41 ± 0.29 ^{e}	8.6 ± 0.14 ^{c,d}	0.357 ± 0.019 ^{d,e}
L9	95 ± 0 ^{a}	9.09 ± 0.12 ^{a}	11.13 ± 0.20 ^{a}	0.463 ± 0.020 ^{a}
B11	83.4 ± 0.67 ^{c,d}	6.32 ± 0.23 ^{e}	8.42 ± 0.17 ^{d}	0.400 ± 0.006 ^{c}
W9	81.7 ± 0.67 ^{c,d}	7.14 ± 0.31 ^{c,d}	8.62 ± 0.38 ^{c,d}	0.337 ± 0.014 ^{e,f,g}
W28	71.6 ± 0.67 ^{f}	3.12 ± 0.26 ^{g}	5.28 ± 0.17 ^{f}	0.207 ± 0.014 ^{h}
LSD	6.565	0.682	0.673	0.0376

3.10. Cluster Analysis

The cluster analysis was performed to categorize the tested strains of this study based on the objectives of this study (Table 6). All the strains were categorized into four groups: the first group of two strains (W28 and ESO-11) comprised of non-selective strains, which reduced the growth of seedlings of all the tested plants; the second group of three strains (T75, T18, and T12) comprised of selective strains, which reduced the growth of little seed canary grass, wild oat, common lambs' quarter, and wheat but not of the broad-leaved dock; the third group of three strains (W9, ESO-8, and T38) comprised of selective strains, which reduced the growth of seedlings of wild oat, broad-leaved dock, and little seed canary grass but not of wheat and common lambs' quarter; and the fourth group of nine strains (T24, $2O_0$, O_010, L9, B11, T19, T42, $7O_0$, and L6) comprised of selective strains, which reduced the growth of seedlings of little seed canary grass, broad-leaved dock, and wild oat but increased the growth of seedlings of wheat. The remaining two strains of this study (T23 and T31) did not suppress the growth of any weed or wheat.

Table 6. Cluster analysis for the selection of bioherbicidal agents based on the response of rhizobacteria in wheat and its associated weeds in agar bioassays. Candidate strains for biological weed control in wheat are indicated in bold.

Category of Strains	Strain	Effects on Weeds and Wheat		
		Inhibition	Promotion	No Effect
Non-selective	ESO-11	All the tested weeds and wheat	–	–
	W28			
Selective and inhibitory to wheat	T12	Wheat, wild oat, and little seed canary grass	–	Broad-leaved dock and common lambs' quarter
	T18	Wheat, wild oat, little seed canary grass, and common lambs' quarter	–	Broad-leaved dock
	T75			
Selective and non-inhibitory to wheat	**T38**	Broad-leaved dock	–	Wheat, wild oat, little seed canary grass, and common lambs' quarter
	ESO-8	Wild oat	–	Wheat, little seed canary grass, broad-leaved dock, and common lambs' quarter
	W9	Wild oat, little seed canary grass, and broad-leaved dock	–	Wheat and common lambs' quarter
Selective and promotory to wheat	T19	Broad-leaved dock	Wheat, wild oat, and little seed canary grass	Common lambs' quarter
	T24	Wild oat	Wheat	Little seed canary grass, broad-leaved dock, and common lambs' quarter
	T42	Wild oat, little seed canary grass, and broad-leaved dock	Wheat	Common lambs' quarter
	$7O_0$			
	O_010			
	L9			
	$2O_0$	Wild oat and little seed canary grass	Wheat and common lambs' quarter	Broad-leaved dock
	L6	Broad-leaved dock	Wheat, wild oat, and common lambs' quarter	Little seed canary grass
	B11	Common lambs' quarter	Wheat	Wild oat, little seed canary grass, and broad-leaved dock
–	T23	–	Wheat	Wild oat, little seed canary grass, broad-leaved dock, and common lambs' quarter
	T31	–	Broad-leaved dock	Wheat, wild oat, little seed canary grass, and common lambs' quarter

4. Discussion

The study of diverse forms of soil-inhabiting microorganisms and their activities may be helpful in resolving many agricultural, environmental, and ecological issues created by unsustainable farming practices. The invasion of weeds in crops reduces their yields, and farmers adopt unsustainable and unhealthy practices to reduce the losses of their crops. The harmful impacts of tillage and chemicals

have been established. Therefore, the present study explored an alternative, inexpensive, sustainable, and environmentally and ecologically safe technique for weed control in crops. It was aimed at finding out the natural mechanisms of rhizobacteria, which function to limit the growth of weeds, alleviate the biotic stress of weeds on crops, and produce a vigorous crop stand. Strengthening such natural processes through augmentation, inoculation, or other processes is required for the development of biological weed control in crops. This may help us to resolve the above-mentioned issues created by conventional control practices [11].

The rhizosphere inhabiting bacteria, which release phytotoxic metabolites in the rhizosphere and result in germination/growth reduction of weeds, are called allelopathic bacteria [18]. The present study is the pioneering work executed in Pakistan, which is aimed at searching such rhizobacteria with their novel characteristics to develop biological weed control. The probability of the existence of such bacteria has been speculated in the rhizosphere of weeds and crops, which are growing together over many years or where the weed invasions occur more frequently [28]. Therefore, we collected the samples of weeds and wheat from areas/fields across the District of Faisalabad, Pakistan, where the weed invasions were more frequent. The findings of this work support the above-mentioned finding of Schippers et al. [28]. They also reported that growth inhibitory rhizobacteria grew, strengthened, and increased their activities in the agricultural crops, where a single crop is grown year after year. It resulted in the reduction of yields of crops. They reported the increase in cyanogenic bacteria and cyanide production in the rhizosphere of potatoes when this crop was continuously grown over a field for 3 years. Their findings increased the importance of crop rotation.

We isolated 393 strains of rhizobacteria from the rhizosphere of five weeds and wheat in this study. These strains were passed through a comprehensive screening process based on the production of phytotoxic metabolites in vitro, suppression of indicator bacteria and plants, in vivo suppression of weeds, and their effects on wheat crops. The protocols followed for these purposes obtained support from the findings of Bakker and Schipper [22], Kremer and Souissi [29], and Kremer [24]. The first test conducted on these strains was the qualitative production of HCN. It was considered a major substance responsible for the growth inhibition of some plants by Kremer and Souissi [29]. This study obtained 22.6% of strains (89) to have produced cyanide at various levels. The distribution of cyanogenic strains in different weeds and wheat was also variable. This was synonymous with the findings of Kremer and Souissi [29]. The proportion of cyanogenic strains in their study (32%) was, however, higher than in our study. This difference might be due to differences in agro-ecological conditions and prevalent agricultural practices. Zeller et al. [19] found that the sensitivity of different weeds and crops to cyanide was variable, and the cyanogenic bacteria might cause suppression of some weeds without imparting harmful effects on the accompanying crop in certain cases. They applied various levels of cyanide to five weeds and wheat and reported that this characteristic of rhizobacteria might be used for the selective suppression of three seeds (*C. jacea*, *G. mollugo*, and *H. murinum*), invading the wheat crop without disturbing the growth of wheat.

The cyanogenic strains of our study were further tested for the production of toxic metabolites using the indicator of sensitive bacteria (*E. coli* strain K12). The relevance of this assay for the screening of rhizobacteria weed control agents was reported by Kremer et al. [30]. We got 21.3% of the cyanogenic strains to suppress the growth of sensitive bacteria. As all the cyanogenic strains did not suppress the growth of sensitive bacteria in this study, one may speculate that the strains inhibiting the growth of bacteria may also have possessed the characteristics of production of some other toxic compounds along with cyanide. This assay indicated that the strains inhibiting the growth of sensitive bacteria might be producing multiple growth inhibitory compounds, collectively termed as antimetabolites, and could be more suitable for testing on weeds and wheat in the next screening studies.

Nineteen strains, obtained from the above screening procedures, were tested on sensitive plant species, i.e., lettuce. The effects of these strains on the growth of the seedlings of lettuce were variable. Some strains inhibited, some promoted, and others remained ineffective. Hence, all the strains inhibiting the growth of *E. coli* did not inhibit the growth of lettuce in our study. This finding agreed

with Kremer et al. [30]. Kremer and Kennedy [31] also reported the growth reduction of lettuce by such rhizobacteria. As the strains tested on lettuce were all cyanogenic in nature, Zermane et al. [32] also reported mixed effects of cyanogenic rhizobacteria on lettuce. The non-inhibition of lettuce by some strains may be due to the non-host interactions, where these strains needed to grow with their host in order to express their characteristics [33]. There also exist differences in the metabolic functions of *E. coli* and lettuce, the former being a prokaryote, and the latter being a eukaryotic plant species. There may also be the difference of compounds, causing antibiosis against bacteria and plants. The results obtained in our study reflected the release of diverse types of metabolites and their functions by these strains, which affected the growth of bacteria and plants. For similar reasons, we tested all the above-mentioned strains on weeds and wheat in the further screening process. This decision in our study has grounds in Souissi and Kremer [34]. They reported the reduction in the growth of weeds by those strains of rhizobacteria, which did not reduce the growth of lettuce. In other words, the growth reduction of lettuce and weeds by rhizobacteria could not be correlated in their study.

Stability or consistency in the characteristics of strains of our study may be evident from the above studies. It increased our reliance on these strains for further studies regarding their effects on weeds and wheat. We found all type of effects of the strains on weeds and wheat, i.e., there were strains inhibitory to all the weeds and wheat, suppressive to one or more weeds and wheat, suppressive to one or more weeds but not to wheat, and suppressive to one or more weeds but promoted the growth of wheat. This array of responses by the strains of allelopathic bacteria has multiple applications if further studies on their characterization and response under natural conditions are carried out. These may be developed for application to control weeds and strengthen crop in poor agricultural systems (selective strains) and control weeds in non-agricultural systems (non-selective strains). The reasons for selectivity may be a difference of tolerance to toxic metabolites in weeds and crop, release of toxic metabolites by these strains only in the rhizosphere of their host plants, the difference in availability of substrates required for the production of toxic metabolites in the rhizosphere of weeds and wheat, a difference of survival, colonization, and establishment in the rhizosphere of weeds and wheat, and difference of mechanisms in the rhizosphere of host and non-host plants [19,20,35]. The findings of our study became more evident when the strains were further characterized by the production of indole-3-acetic acid, exopolysaccharides, siderophores, catalases, chitinases, oxidases, and P solubilization. The most prominent strains were identified as pseudomonads. The effects of the five most efficient strains on weeds and wheat were tested under axenic conditions in Abbas et al. [36]. The strains inhibiting one or more weeds and promoting wheat may be more successful for weed control under natural conditions. These may strengthen the weak crop plants, increase their competitive ability, and, hence, increase the scale of weed control by allelopathic bacteria. The non-selective strains inhibitory to wheat may be tested for their effects on other crops to explore opportunities for their application in other cropping systems. The efforts on augmentation of effects of allelopathic bacteria under natural conditions may be helpful to realize the dream of biological weed control. The strains of allelopathic bacteria obtained from this study can be further tested for their effects on weeds and wheat under field conditions. Further efforts may be required to improve their efficiency of weed control under natural conditions. Application methods of allelopathic bacteria may also be needed to be optimized. This will produce a bioherbicide for the control of weeds in an environmentally friendly and sustainable manner.

5. Conclusions

The rhizosphere of five weeds and wheat, growing in areas of high weed invasion, was explored for the allelopathic bacteria. A large collection of strains of rhizobacteria was passed through a comprehensive screening process for this purpose. We got 22.6% strains cyanogenic in nature, 21.3% of which (19 strains) inhibited the growth of sensitive bacteria. These strains were applied to lettuce, which showed mixed effects. These strains were later tested on four weeds and wheat. We got strains inhibitory to all these weeds (eight for the broad-leaved dock, seven for wild oat, eight for little seed canary grass, and three for common lambs' quarter). They reduced the dry matter of these weeds from

23.1 to 68.1%, 38.5 to 80.2%, 16.5 to 69.4%, and 27.5 to 50.0%, respectively. Only five of these strains were inhibitory to wheat; the others either remained neutral (five strains) or improved the growth of wheat (nine strains). These strains offer opportunities for the development of biological weed control.

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