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Screening fluorescent pseudomonads isolated from wheat rhizosphere for plant growth- promoting and salt tolerance properties

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ABSTRACT: 30 fluorescent pseudomonads were isolated from wheat rhizosphere in Bushehr province, Iran. Strains were screened for the key features of plant growth promotion including production of hydrogen cyanide and siderophore and inorganic phosphate solubilization under in vitro conditions. Then, superior strains were evaluated for production of indole acetic acid (IAA). Results revealed that 100% of isolates were able to produce siderophore, 74.44% produced hydrogen cyanide and 96.66% were capable of solubilizing inorganic phosphate. All superior PGPR strains evaluated for IAA production were able to produce the metabolite. 30 Pseudomonas strains isolated in this study, were evaluated for tolerance to salinity levels of 200, 400 and 600 mM sodium chloride (EC equivalent, respectively, 9/28, 3/47 and 2/61 ds/m). Results showed that all strains could tolerate 400 mM NaCl concentrations and had a weak growth at 600 mM (classified as semi-tolerant), but none of them could grow strongly on 600 mM concentration.

Keywords: Wheat, salt stress, siderophore, hydrogen cyanide, fluorescent pseudomonads

INTRODUCTION

Wheat (*Triticum aestivum* L.) belongs to Poaceae family, which is one of the largest families in flowering plants and consists of 600 species in 450 genera. In recent years, due to the environmental pollution crisis, especially soil and water resources pollution, which have lead to the infection of food sources and threaten human health, wide efforts have been started. In sustainable agricultural systems, using bio-fertilizers especially in poor soils is of particular importance in enhancing the performance and maintaining high soil quality (Sharma, 2012).

Plant growth promoting rhizobacteria (PGPR) which are found in the rhizosphere of numerous host plants, are able of produce plant hormones, fix nitrogen, uptake nutrients from soil and produce secondary metabolites against plant pathogens, and affect plant growth (Vessey, 2003). The ability of these bacteria to increase nutrient absorption, developing plant root system and controlling plant pathogens, lead to the widespread use of them and it is expected that in the future they can replace a portion of chemical fertilizers (Compant *et al*, 2005, Tenuta, 2005). Rhizosphere inhabiting Fluorescent pseudomonads are one of the most important components of the rhizosphere bacterial populations (Benizri *et al*, 2001). Different species of the genus Pseudomonas have been effective in controlling plant pathogenic agents through various mechanisms including siderophore and hydrogen cyanide production, synthesis of antibiotics, production of plant hormones, increasing phosphorus uptake by plants, nitrogen fixation and synthesis of enzymes that regulate ethylene and increase plant growth (Abdul Jaleel *et al*, 2007).

Microbial siderophores are relatively large organic molecules with a molecular weight of about 1,000 to 1,500 Daltons that have a great affinity for binding with Fe^{3+} (Milagres *et al*., 1999). Due to specific receptors on bacterial cell membrane, they are able to absorb the iron-siderophore complexes. Due to the siderophore production and competition for various substrates, especially iron, Pseudomonas species are inhibitory to many plant pathogens (Buysens *et al*., 1996).

The ability of various strains of soil bacteria to dissolve the insoluble inorganic phosphates have been mentioned in various studies (Goldstein, 1986). Plant growth- promoting rhizobacteria (PGPR) including fluorescent pseudomonads, are able to solubilize poorly soluble inorganic compounds such as phosphorus and zinc. These bacteria reduce rhizosphere soil pH via different mechanisms, including the production of organic acids, inorganic acids and proton secretion and in result increasing the availability of nutrients for plants (Sundra *et al*., 2002). The phosphate solubilizing bacteria (PSM) as a complementary component of the phosphorus cycle can release insoluble phosphorus through various mechanisms (Zahir *et al*., 2009).

Production of hydrogen cyanide (HCN) is one of the most important mechanisms of biological control of plant pathogens by PGPR (Nadine *et al*., 2010). Hydrogen cyanide is a secondary metabolite produced by some Gram-negative bacteria including fluorescent pseudomonads. HCN production by pseudomonads can increase plant growth by controlling plant pathogens (Castric, 1977). It is suggested that the ability to generate HCN by PGPR is a potential for biological control of weeds and a mechanism that can be used as a new approach to strengthen and stimulate plant growth and increase crop yield (Antoun and Kloepper, 2001, Kremer and Suissi, 2001).

Besides, some PGPR strains are capable of stimulating plant growth by changing the concentration of known phytohormones (Glick, 1995). Among phytohormones, auxin is the first hormone recognized by Darwin in 1880. Elongation of plant cells, phototropism, geotropism, rooting and root elongation, and finally stimulating ethylene production, followed by the evolution and maturation of fruits are considered as known effects of auxin in plants. Indole-3-acetic acid (IAA) is the most common natural auxin and has wide effects on plant physiology (Bric *et al*., 1991). Increasing the concentration of IAA in the rhizosphere region results in accelerating growth and development of the root system of plants. This in turn increases root exudates and IAA signals, and eventually causes production of large amounts of IAA and increases plant yield (Lambrecht *et al*., 2000).

Salinity is one of the first environmental stresses that plants face, but the significance of this stress over other environmental stresses, is that salinity has a permanent effects on plants. Unlike other environmental stresses that plants encounter as part of their growth period, salinity affects the entire period of plant growth (Farrokhi and Galeshi, 2005). One of the reasons for the decline or lack of growth under abiotic stresses such as salinity, is the accumulation of ethylene in plant tissues. One way to reduce the negative effects of salinity is to apply plant growth promoting bacteria (PGPR) which are tolerant to salinity stress (Bacilio *et al*., 2004). The main mechanism of these bacteria to facilitate plant growth is reducing the amount of ethylene (Mayak *et al*., 2004). It is reported that rhizobacteria increase plant resistance to salinity through improving root system, decreasing sodium absorption and increasing the expression of genes responsible for providing plant resistance to environmental stresses (Rogers and burns, 94, Ashraf *et al*., 2004,). Studies show that PGPRs provide nutrients to the plants and prevent the osmotic stress triggers by the addition of chemical fertilizers to saline land, while use of chemical fertilizers in the ecosystem, only damages the soil physical, chemical and biological structure, but also but also affects the quality of plant products (Koochaki *et al*., 2008).

The aim of this study was to evaluate plant growth promoting characteristics of fluorescent pseudomonads isolated from wheat rhizosphere in Bushehr province, Iran, and determining their tolerance to salt stress under in vitro conditions. It is expected that at least some of these strains could stimulate plant root growth, enhance nutrient absorption and increase wheat yield.

MATERIALS AND METHODS

A. Isolation and relative identification of fluorescent pseudomonads

Rhizosphere soil samples were collected from different wheat fields in Bushehr province, Iran and transferred to the lab. Bacteria were isolated from rhizosphere using serial dilution method. Nutrient Agar (NA) and King?S medium B (King *et al*., 1954), were used for isolation and purification of bacteria. The production of fluorescent pigment by fluorescent pseudomonads was confirmed using S1 medium under UV light (366 nm).

Phosphate solubilization test. Solubilization of phosphate was tested on solid Sperber medium containing 2.5 g three calcium phosphate (Sperber, 1958). 24-hour-old bacterial isolates were grown on solid medium and the formation of transparent halos around each bacterial colony showed solubilization activity. The resulting halos zone around the colonies after incubation for 7 days showed the presence of bacterial activity in solubilization of mineral phosphate. The halo diameter was determined after a week of incubation.

Production of siderophore type pyoverdine. Quantification of pyoverdine production by bacterial isolates was examined by spectrophotometry method described by Castaneda *et al*. (2005). 100 µl of 24-hour-old bacterial strains grown in liquid succinate medium was transferred to erlen-meyers containing 40 ml of fresh succinate medium. Bacteria were incubated in shaker incubator, for 40 hours at 27°C at 120 rpm. Bacterial cells were centrifuged at 10,000 g for 10 minutes. The absorption of supernatant was measured at 400 nm with a spectrophotometer. Data were converted to moles/ liter using the formula A = BC. A= Absorption; = Molar absorption coefficient; B= Cuvette diameter and C = material concentration.

Hydrogen cyanide production. Screening of bacterial isolates for the production of hydrogen cyanide (HCN) was performed according to the method described by Alstrome (1989). The medium used is NA containing 4.4 g per liter of glycine. The production of cyanide was detected using a solution of cyanide detection solution (CDS) containing 2 g of picric acid and 8 grams of sodium carbonate dissolved in 200 ml of sterile distilled water.

Pieces of filter paper the size of 1 x 1 cm was immersed in a solution of CDS and placed on the bottom of a petri dish, incubated at room temperature for 4 days. Discoloration of the filter paper from orange to brown after incubation was considered as microbial production of cyanide.

IAA production. Production of auxin indole -3-acetic acid (IAA) by bacteria was tested using LB and Salkowski reagent. Isolates were grown on LB equipped with L-tryptophan (5 mM) at 30ºC for 24 hours. The supernatant was taken after centrifugation. One ml of the supernatant was added to two ml of Salkowski reagent (12 g l^{-1} $FeCl_3$ in 429 ml l^{-1} H_2SO_4) and incubated at room temperature for 2.5 hours. The intensity of pink colour developed was read at 530 nm using a UV-VIS spectrophotometer (Bric *et al*., 1991). From a standard curve prepared with known consentration of IAA, the quantity in the culture filtrate was determined and expressed as mg l^{-1}. The test was performed only for five strains that were superior in previous plant growth stimulating tests (Production of HCN and siderophore and solubilizing phosphate).

Strain tolerance to salinity stress under in vitro conditions. The pure culture of all isolates was streaked on NA medium containing 0, 200, 400 and 600 mM of NaCl. 3 replications were considered for each isolate. Bacteria were incubated at 28 ºC and colony growth was monitored after 120 hours. Based on the bacterial growth in different salt concentration, and its comparison to other strains, isolates were classified as sensitive, semi-sensitive, semi-tolerant, tolerant and very tolerant.

Statistical analysis. Each experiment was conducted in completely randomized design with three replications. The experimental data were analyzed statistically using SAS program version and means were separated by Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

The main goal of developing biotechnology regarding plant growth-promoting rhizobacteria is to increase the population of effective bacteria which may help the development of sustainable agriculture (Adesemoye and Kloepper, 2009). Studies have shown that fluorescent pseudomonads are present actively in most agricultural soils and are responsible for declining some root diseases caused by soilborne plant pathogens and increasing crop yield.

Of 30 isolates selected for determining PGPR characteristics, all had at least one of the PGPR traits. 100% were able to produce siderophore, 74.4% were HCN producers and 96.6% percent were capable of solubilizing phosphate. Four out of five 5 superior strains evaluated for the production of IAA were positive for this trait. Cattelan *et al*. (1999) isolated and studied 116 strains from soybean rhizosphere, of which 22 isolates had PGPR characteristics. Sarcheshmeh-Pour *et al*. (2010) examined 104 isolates from pistachio rhizosphere, of which 80 percent had at least one plant growth stimulating trait. Antoun *et al*. (1998) reported that of the 266 strains of Rhizobium bacteria 83 percent produced siderophore, 58 percent were IAA producers and 54 percent solubilized phosphate.

Results revealed that all 30 Pseudomonas isolates were able to produce siderophore type pyoverdine. However, strains were significantly different based on siderophore production ($p \leq 0.01$). Of all isolates, 13 strains produced more than 5 µM/ml. Isolates Wh-ND2, W13, WBO-3 and WKZ1-93 produced the highest amount of siderophore, respectively.

Kumar *et al*. (2014) showed that siderophore production was exhibited by 20% of 58 isolates of different genera of soil bacteria. Similarly to our findings multiple PGP activities among PGPR have been reported by other studies (Gupta *et al*., 1998; and Dey *et al*., 2004). Several researches have shown that production of siderophore by PGPR is the most effective mechanism in controlling plant pathogens (Mullen, 1998; Diaz *et al.*, 2002; and Dey *et al.*, 2004). The ability to produce siderophores by microorganisms in improving iron availability to plants was also reported by other researchers (Kloepper *et al*. 1980, Bar-Ness *et al*., 1992; Rroco *et al*., 2003) In the study performed by Sarcheshme-Pour *et al*. (2010), 46% of isolates produced siderophore on CAS-Agar and the ratio of halo diameter to colony diameter decreased over the time. The results of Soltani *et al*. (2008) showed that all *P. fluorescens* isolates produced siderophore and none of Flavobacterium isolates had this ability.

Our results revealed that most of the isolates were able to produce HCN. Of 30 isolates, isolates WKZ1-93, WT1-82, WKZ1-100, WBO2-5, Wh-m90, 2-79, WKZ2-15 and Wkz1-105 produced more HCN than others. Isolates WB, Wh-E23, WB1-8, WBO-3, WT1-56, WKZ1-10, W15, W13 and Wh-m^2 were unable to produce this metabolite (Table 2).

Hydrogen cyanide (HCN) is of secondary metabolite of many microorganisms which can be produced directly from proline, glycine or cyanogenic glycosides. HCN production by Pseudomonas strains depends on availability and the amount of Fe^{3+}.

Table 1: Production of siderophore type pyoverdine (µM/ml) by fluorescent pseudomonads isolated from wheat rhizosphere.

No.	Isolates	Production of Siderophore type pyoverdine (µM/ml)	No.	Isolates	Production of Siderophore type pyoverdine (µM/ml)
1	P-101	2.34kl	16	WB1-8	5.1ef
2	Wh-DN2	3.15j	17	WT1-82	3.7i
3	WKZ-88	4.36h	18	WKZ1-100	3.06j
4	WKZ2-5	1.5no	19	WBO-3	8.5a
5	WKZ1-93	7.1 c	20	WT1-56	1.4no
6	Wh-jkh	1.13o	21	WKZ1-4	1.53no
7	WB	5.03fg	22	WKZ1-10	6.03d
8	Wh-m9	2.73kj	23	WBO2-5	6.26d
9	Wh-m90	2.53kl	24	W4	1.8mn
10	WT1-25	7.66 b	25	W15	6.16d
11	Wh-E23	4.63gh	26	W13	8.8 a
12	WB1-7	5.18ef	27	Wh-m2	3.7i
13	WB1-20	2.2lm	28	2-79	5.5e
14	WT1-26	5.26ef	29	WKZ1-88	1.76n
15	Wkz1-105	1.26o	30	WKZ2-15	6d

*means with different letters are significantly different ($p \leq 0.01$)

Table 2: Production of HCN by fluorescent pseudomonads isolated from wheat rhizosphere.

No.	Isolates	Production of HCN	No.	Isolates	Production of HCN
1	P-101	2b	16	WB1-8	0d
2	Wh-DN2	2b	17	WT1-82	3a
3	WKZ-88	1c	18	WKZ1-100	3a
4	WKZ2-5	2b	19	WBO-3	0d
5	WKZ1-93	3a	20	WT1-56	0d
6	Wh-jkh	2b	21	WKZ1-4	2b
7	WB	0d	22	WKZ1-10	0d
8	Wh-m9	2b	23	WBO2-5	3a
9	Wh-m90	3a	24	W4	1c
10	WT1-25	2b	25	W15	0d
11	Wh-E23	0d	26	W13	0d
12	WB1-7	2b	27	Wh-m2	0d
13	WB1-20	1c	28	2-79	3a
14	WT1-26	2b	29	WKZ1-88	1c
15	Wkz1-105	3a	30	WKZ2-15	3a

*HCN production (0-3) was based on 0=no HCN production, 1= the least HCN production, 2= average HCN production and 3= the most HCN production, **means with different letters are significantly different ($p \leq 0.01$)

On the one hand, the metabolite is toxic to pathogenic fungi, and on the other hand, its production by bacteria causes capillary root formation (Schippers *et al*., 1987). In the current study, 22 Pseudomonas isolates were able to produce hydrogen cyanide, which was variable from low to high. The results Azarmi *et al*. (2014) showed that 75% of evaluated fluorescent pseudomonads were able to produce HCN which ranged from relatively- low to high. Phosphate solubilization ability is marked by the formation of transparent halos around the colony bacteria in media containing tricalcium phosphate (Ca_3PO_4). The Pseudomonas strains studied in this research were significantly different based on solubilizing phosphate ($p \leq 0.01$). Our results revealed that of 30 rhizosphere Pseudomonas isolates, 96.6% were able to solubilize phosphate and isolates were different regarding this ability. All isolates but WB were capable of solubilizing phosphate. The isolates that showed the best capability of solubilizing phosphate were WBO2-5, WKZ1-88 and WBO-3 with halo diameter of 30.17, 28.67 and 25.67 mm, respectively.

Table 3: Phosphate solubilization by fluorescent isolates under in vitro conditions.

No.	Isolates	Phosphate Solubilization (halo diameter in mm)	No.	Isolates	Phosphate Solubilization (halo diameter in mm)
1	P-101	15.33 gfjlekhi	16	WB1-8	14 gjlmkhi
2	Wh-DN2	14.33 gjlkhi	17	WT1-82	17.67 gfdehi
3	WKZ-88	23.83 bdac	18	WKZ1-100	12.67 jlmkhi
4	WKZ2-5	14.67 gfjlkhi	19	WBO-3	25.67 bac
5	WKZ1-93	12.33 jlmkhi	20	WT1-56	22 bdec
6	Wh-jkh	16.33 jlmki	21	WKZ1-4	12 jlmkhi
7	WB	0m	22	WKZ1-10	11.33 bdc
8	Wh-m9	21.5 fdec	23	WBO2-5	30.17a
9	Wh-m90	9.17 lmk	24	W4	10 jlmk
10	WT1-25	7.33 gfjehi	25	W15	16 gfjekhi
11	Wh-E23	9 lmk	26	W13	10 jlmk
12	WB1-7	19.83 gfdec	27	Wh-m2	8.33 lm
13	WB1-20	11.5 jlmkhi	28	2-79	15 gfjlkhi
14	WT1-26	17.33 gfdehi	29	WKZ1-88	28.67 ba
15	Wkz1-105	12.67 gfdeh	30	WKZ2-15	12.67 jlmkhi

*means with different letters are significantly different ($p \leq 0.05$)

Jeon *et al*. (2003) showed that three strains of *P. fluorescens* in PKV medium containing 5 g/l tricalcium phosphate could solbilize 458.3, 447.6 and 427.7 µg/ml of insoluble phosphate. Ahmad *et al*. (2006) studied 72 various strains of bacteria and found that 55.5% of Pseudomonas strains and more than 80% of Bacillus strains were phosphate solubilizers. Sarcheshmeh-Pour *et al*. (2010) reported that all superior phosphate solubilizing strains belonged to the genus Pseudomonas and had more efficiency due to their ability to reduce pH. Sundra *et al*. (2002) reported that PSB, reduce soil pH through organic acids production and thereby cause greater access to elements such as potassium. The study of Rafiei *et al*. (2013) on the measurement of solubilization of inorganic phosphates by Flavobacterium strains on solid Sperber medium showed that of 44 strains, 16 isolates could not grow. The remaining isolates (28 isolates) the index of inorganic phosphate solubilization (the ratio of colony diameter) increased over the time. Meunchang *et al*. (2006) isolated 168 PGPRs and showed that 62 isolates (37%) were capaable of phosphorus solubilization.

The ability of the bacterial isolates to produce IAA was detected by the development of pink colour after the addition of salkowski reagen to the culture. In this study, all five isolates were able to produce IAA growing in LB medium amended with l-tryptophan. Maximum IAA production was recorded in isolate WB1-7 (7.52 mg/l) as compared to other isolates. The minimum amount of IAA production was recorded in isolate Pf-101 (2.08 mg/l) (Table 4).

Table 4: Production of indole acetic acid (IAA) by bacterial strains.

Bacterial strains	IAA production (mg/l)
P101	2.08b
WBO-3	2.51b
2-79	2.77b
WKZ1-93	3.16b
WB1-7	7.52a

*means with different letters are significantly different ($p \leq 0.01$)

Indole acetic acid is the most important kind of auxine, which is produced through L-tryptophan metabolism, which is produced by plants and microorganisms including bacteria, fungi and algae. Fluorescent pseudomonads are the most abundant and the most significant auxin producers which have a great impact on plant growth (Khaki-Pour *et al*., 2008). Sarcheshmeh-Pour *et al*. (2010) revealed that of the 104 isolates examined, 47% were IAA producers. The auxin production was variable of a minimum of 2.67 to a maximum of 34.3. In the study performed by Soltani *et al*. (2008) it was shown that auxin production was variable in *P. fluorescens* isolates from 1.3 to 4.5 and from 0.27 to 3.12 µg/ml in Flavobacteria. Alikhani *et al*. (2007) reported that from 220 Iranian Rhizobium isolates, 74.1% produced IAA.

Ahmad *et al*. (2006) found that from 72 isolates of rhizosphere bacteria in India, 80% of Pseudomonas spp. and 20% of Bacillus spp. were IAA producers and that auxin production in 11 strains of *P. fluorescens* studied ranged from 5.34 to 22.44 µg/ml. In another study Kesaulya *et al*. (2015) isolated 70 strains from potatoe rhizosphere of from the 70 strains, of which 36 strains were able to produce IAA; HB8 strain produced the highest amount of auxin (5.8 mg/l). In this regard, Asghar *et al* (2004) studied auxin production in 53 isolates belonging to different genera and found that the bacteria were able to produce the amount of 3.1 to 7 mg auxin and Pseudomonas putida the most effective isolate. Research of Soltani Toola-Rood *et al*. (2008) on auxin production by 25 florescent Pseudomonas isolates showed that 25 isolates were capable of auxin production and average production rate was 2.44 µg/ml. Results showed that all strains were able to tolerate 200, 400 and 600 mM of NaCl and only WKZ-88, Wkz1-105 and WKZ1-88 had the least tolerance to 600 mM concentration. Strains was classified into 5 groups based on their growth on different salinity levels (Table 5).

Table 5: Tolerance of fluorescent pseudomonas isolates to different levels of salinity under in vitro conditions.

Degree of tolerance	Growth conditions	Number of isolates
Sensitive	weak to relatively low growth at 200 mM concentration	27
Semi-sensitive	average to high growth at 200 mM concentration	3
Semi-tolerant	weak to relatively low growth at 400 mM concentration	30
Tolerant	average to high growth at 400 mM and weak to relatively low growth at 600 mM concentration	30
Very tolerant	average to high growth at 600 mM concentration	-

Most of the Pseudomonas strains in this study could tolerate the maximum level of NaCl concentration (400-600 mM). Increasing salinity concentration decreased the colony mass of some isolates. Cordovilla *et al*. (1999) showed that the compatibility of bacteria to high salinity levels is due to the increase in intercellular potassium and glutamate levels. It is suggested that pre-treatment of seeds with PGPRs reduces destructive effects of salt stress which is because of the ability of bacteria to produce phytohormones and increasing root ability to absorb water. Kumar *et al*. (2014) examined 58 isolates of *Pseudomonas*, *Bacillus* and Azotobacter for salt tolerance and revealed that all isolates were tolerant to 3% NaCl and isolates Azt5, Bc1 and Bc3 tolerated salt even at 7% NaCl. Rangarjan *et al*. (2002) studied 256 Pseudomonas strains for salt tolerance and showed that only 36% could grow on 4.5% NaCl concentration.

In this research plant growth promoting traits of Pseudomonas strains were very significant and because of the high tolerance of these strains to salt stress it is expected that after performing greenhouse experiments they can be applied on seeds or in soil drench to improve the nutritional condition and wheat tolerance to salt stress. It is believed that isolation and studying indigenous bacterial strains which are compatible to environmental conditions results in the production of inoculums which are more efficient to the crops of that region comparing to foreign strains (Fisher *et al*., 2007).

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