THE POTENTIAL OF CULTIVATION CONDITIONS IN THE BACTERIOSTATIC EFFECT OF SOME PGPR ISOLATES AGAINST SOME PATHOGENS

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The bioactive metabolites production was studied to optimize the media composition and cultivation conditions for bacterial isolates having antibiosis efficiencies and were identified based on biochemical characteristics and 16S rRNA gene sequencing. The effect of different carbon sources (glucose, glycerol, lactose, mannitol and sucrose), nitrogen sources (ammonium chloride, ammonium nitrate, L-glutamic acid, peptone and sodium nitrate), pH values (4.0-9.5), incubation time (24, 48, 72, 96 and 120 h), temperature (15, 20, 25, 30, 35, 40 and 50ºC) and salinity (1.5, 2.5, 3.5, 4.5, 5.5 and 6.5%) on the antibacterial activity were investigated using agar diffusion assay and detected by the zone of inhibition sizes. The antibacterial activity of the tested plant growth promoting rhizobacterial (PGPR) strains, in liquid static culture, strongly impacted by the cultivation conditions, which support considerable antibiosis capabilities for the tested PGPR antagonist's strains, *Bacillus altitudinis*, *Brevibacillus brevis*, *Paenibacillus xylanexedens* and *Stenotrophomonas maltophilia*, against the test culture of *Eschericia fergusonii*. The maximum activity was observed at neutral nutrient media (pH 7.0) enriched by glucose as carbon source, L-glutamic acid as nitrogen source with or without 1.5% NaCl salinity and after incubating at 30ºC for 48 h, with over 30 mm zone of inhibition against *Eschericia fergusonii*.

Keywords: culture media, antibiosis, promotion, Rhizobacteria

INTRODUCTION

Kloepper described plant growth promoting rhizobacterial (PGPR) as ‘‘Soil living bacteria that colonize or incorporate plants roots and improve the

growth of plants" in late 1970s. Plant health promoting rhizobacteria (PHPR), and nodule promoting rhizobacteria (NPR), are other definitions for PGPR (Kloepper et al., 1989 and Gray and Smith, 2005).

Rhizosphere, at where PGPR are attached, plays a key role in the ecological and environmental soil - plant-microbe interactions (Hayat et al., 2010). PGPR could be identified as environmentally friendly biological control agents, with no harmful effect on users or consumers therefore have promising biotechnology applications (Abdelaziz et al., 2018). PGPR smooth the way for plant growth and progress with a variety of advantageous effects, more so obvious when plants are grown in condemnatory environmental conditions, such as diminishing metal phytotoxicity by adapting metal bioavailability in soil to metal translocation inside the plants, also many of PGPR are able to oxidize hydrocarbons resulting an enhancing in the biodegradation activity for plants. It can also replace chemical fertilizers that impact negatively on environment since they are capable to alleviate the effects of biotic stresses like drought and salinity (Vocciante et al., 2022)

PGPR include a wide range of species have been reported as plant growth promoters and antagonize plant pathogens, for instance, *Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Burkholderia, Enterobacter, Flavobacterium, Klebsiella, Mesorhizobium, Pseudomonas, Rhodococcus, Streptomyces, Serratia* (Ahmad et al., 2008 and Tariq et al., 2014). PGPR produce growth inhibitors as, antibiotics, lytic enzymes, siderophores and bacteriocins, and increase systemic resistance of host plant. The antagonistic effects of PGPR include competition, parasitism (Paul et al., 2001 and Anith et al., 2004).

Antibiotics discovered in the initial of 20th century and have been produced by a variety of microorganisms as secondary metabolites (Schallmey et al., 2004). Most peptide antibiotics are produced by *Bacilli*, target the bacterial cell membrane directly (Browne et al., 2020),

Bacillus species, Gram-positive PGPR, have antimicrobial activity and are capable of synthesize a wide range of antimicrobial compounds (Nihorimbere et al., 2010) such as circulin, polymyxin, colistin, fengycin and iturins (Romero et al., 2007 and Maksimov et al., 2011), bacillomycin (Chen et al., 2007), bacillibactin (Dertz et al., 2006), and lipopeptides (Ongena and Jacques, 2008) besides inducing systemic resistance (ISR) against an invader such as a pathogen or parasite. In addition, *Bacillus* produces bacteriocins which have a broad range of inhibitions against Gram-positive and Gram-negative bacteria, fungi, and yeasts (Abriouel et al., 2011). The phylum Bacillota, or "Firmicutes" which originated from the Latin words for "tough skin," include *Bacillus* and *Paenibacillus* species, most of which, the cell wall structure is Gram-positive and have potentiality to produce varieties of structurally various antimicrobial compounds (Olishevska et al., 2019).

Bacillus species at a regular pace become focused globally on biological control to their adequate simple nutrition demands, fast replicating,

endospores, resistance against stresses plus producing a variety of antibacterial compounds against multiple pathogens (Pertot et al., 2013)

Paenibacillus, which was clarified in 1993 (Ash et al., 1993), originated in the rhizosphere of several plants in soil, and most strains of the genus are form resistant spores, can solubilize phosphate, and get through arid climates and bears both biotic and abiotic stresses (Fahsi et al., 2021). Also, capable to produce a diverse of antimicrobial compounds as biological control agents. The antimicrobial compounds produced by *Paenibacillus* have a wide spectrum against fungi, Gram-positive and Gram-negative bacteria. *Paenibacillus* showed antagonistic activities against *Escherichia coli*, *Staphylococcus aureus* and more (Wu et al., 2010 and Guo et al., 2012). Most of the secreted compounds are peptide antibiotics like saltavalin, gatavalin, and polymyxins (Li et al., 2007).

Brevibacillus (formerly, *Bacillus brevis*) is widely spread in diverse environments like dust and rocks even in aquatic environs, predominantly in agricultural soil (Kim et al., 2009 and Sharma et al., 2012) and known as substantial source for bioactive antibacterial and antifungal peptides. It can secrete constitutionally various secondary metabolites with wide antibiotic spectra such as loloatin, tauramamide, chitinase and germicidin, firstly known antibiotic, and some other strains produce probiotics commercially (Krachkovskii et al., 2002; Desjardine et al., 2007; Mogi and Kita, 2009; Zhao et al., 2012 and Yang and Yosef, 2018).

Stenotrophomonas maltophilia is a common nonparasitic free-living bacterium, previously known as *Pseudomonas maltophilia* (Palleroni and Bradbury, 1993) and now recognize as an emanating opportunistic pathogen (can be found in healthy hosts and become virulent with impaired immunity) (Deredjian et al., 2016). It is a non-endospore forming, Gram-negative bacterium and less active against Gram-positive comparing with Gram-negative bacterial strains (Sarkar et al., 2022).

Stenotrophomonas maltophilia has been isolated from various environments, such as agricultural soils (Sturz et al., 2001), plant rhizosphere, soil, and water (Palleroni and Bradbury 1993; Bollet et al., 1995 and Romano et al., 1997). It was mentioned that it occurs in extreme ecological environments, deeply in seas or at great height altitudes (Romanenko et al., 2008 and Flores et al., 2009), sludges and contaminated sites (Dungan et al., 2003 and Matyar et al., 2008), from the interior of plant tissues (Taghavi et al., 2009) and industrial wastes (Franco et al., 2005). *Stenotrophomonas maltophilia* produces phytohormones (Peralta et al., 2012), it has antagonistic an effect with capability to secrete secondary metabolites against pathogenic fungi and exoenzymes like proteases, chitinase and gluconases (Berg et al., 1996 and Zhang et al., 2001), and used in bioremediation (Caliz et al., 2012 and Meyer et al., 2012).

The components of the growth medium have a significant effect on microbial growth and metabolites production (Rana and Sahgal, 2017), the

production of antibiotic substances and its effect (Wood and Tveit, 1955). The exact combination of nutrients raised growth and antagonistic of inhibitor agents (Slininger et al., 1996). At industrial production, components character and accumulation of the fermentation setting may control antibiotic (Sanchez and Demain, 2002).

For each new strain, it is necessary to determine individual cultivation condition, Jiménez-Delgadillo et al. (2018), stated that the inhibitory effect occurs mainly in the stationary phase and directly influenced by cultivation conditions. The composition of antagonistic metabolites is affected qualitatively and quantitatively by the change in the growth medium acidity (Sidorova et al., 2020). Antagonistic activity according to enzyme production depends on nutria pH (Shanmugaiah et al., 2008).

This study was conducted to evaluate the optimum laboratory cultivation conditions for the synthesis of antimicrobial metabolites by antagonistic strains and pointing out the effect of salt stress on the isolate's antagonistic activity. The novelty of this work is composed of the multifunctional plant growth promotion and biocontrol potential of antagonist soil bacteria in connection with cultivation conditions and media composition.

MATERIALS AND METHODS

1. Bacterial Isolates

Four PGPR bacterial strains were sed in this study; *Bacillus altitudinis*, *Brevibacillus brevis*, *Paenibacillus xylanexedens* and *Stenotrophomonas maltophilia* and isolate of *Eschericia fergusonii* previously isolated from El-Qantara Shark soil rhizosphere irrigated with El-Salam canal water and identified according to the biochemical properties and 16S rRNA gene sequencing (Helal et al., 2020).

2. Plant Growth Promoting Substance Screening of PGPR Isolates

Indole acetic acid (IAA), gibberellins (GA) production were determined according to Shindy and Smith (1975) and polysaccharides were determined according to Emtiazi et al. (2004).

3. Antagonism Screening of PGPR Isolates Against *Escherichia Fergusonii*

According to Liu et al. (2006), agar diffusion method, from suspension of pathogenic culture, one hundred microliters per each was added to Petri dishes with nutrient agar medium, mixed well, and solidified. From PGPR suspensions, 5 mm discs were moisturized and seeded on the agar plates, incubated at 30^{o}C for 24 h, and the inhibition zone diameters were measured.

4. Cultivation Conditions Affecting Antibiosis of Bacterial Isolates

Escherichia fergusonii was chosen for experiments as a form of bacteria generally exist in the disease environs. The antagonistic outcomes of

the four PGPR strains grown under different cultivation conditions were monitored against the pathogen *Escherichia fergusonii*. The antagonist's liquid cultures were centrifuged at 8000 rpm for 10 min. In the cell-free culture filtrates, filter paper discs (5 mm diameter) were soaked and distributed on nutrient agar plates previously seeded with the pathogen.

5. Carbon Source

The PGPR-nutrient broth culture media; enriched with glucose, glycerol, lactose, mannitol, or sucrose (1%, w/v), were tested. The inhibition zone diameters of the pathogen due to antagonists were measured after 24 h incubation at 30ºC.

6. Nitrogen Source

The inhibition zones of *Escherichia fergusonii* in the presence of the bio-agents, previously cultivated in nutrient broth medium received 0.5% (w/v) of either ammonium chloride, ammonium nitrate, L-glutamic acid, peptone, or sodium nitrate, were measured after 24 h incubation at 30ºC.

7. Incubation Temperature

The bacterial isolates grown in nutrient broth medium were incubated at 15, 20, 25, 30, 35, 40 and 50ºC and were evaluated for antibiosis after 24 h incubation.

8. Incubation Period

The lethal impacts of the antagonists incubated for 24, 48, 72, 96 and 120 h at 30ºC towards *E. fergusonii* were assessed.

9. Hydrogen Ion Concentration (ph)

The suppression of the pathogen due to the PGPR bioagents grown in the culture media of pH values of 4.0-9.5 was recorded after 24 h incubation at 30ºC.

10. Salinity Level

The pathogen suppression was scored due to PGPR cultivated in nutrient broth medium supplemented with NaCl in the concentrations of 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5%.

RESULTS AND DISCUSSION

1. Production of Plant Growth-Promoting Substances (PGPS) by PGPR Antagonists

All isolates were able to produce IAA, GA and polysaccharides. *Bacillus altitudinis* was the highest isolate in the production, by 2030 (µg/l), 40.1(mg/l) and 55.8 (mg/l) for IAA, GA, and polysaccharides, respectively (Table 1). *Paenibacillus xylanexedens* was the lowest one in producing GA and polysaccharides by 26.9 and 11.2 (mg/l) respectively, while *Brevibacillus brevis* produced the lowest amount of IAA by 730 µg/l, comparing with the

other tree assessed isolates (Table 1). This finding is an agreement with the previous literature confirmed that PGPR are capable of secreting a wide spectrum from promoting plant substances, as mentioned by Sivasakthi et al. (2014), Huang et al. (2016) and Xiang et al. (2017). Bacteria, which put together antimicrobial activities and PGP abilities, may play a part in sustainable agricultural development.

Table (1). Plant growth promoting substances produced by PGPR strains in nutrient broth medium.

Plant growth promoting substance	***Bacillus altitudinis***	***Brevibacillus brevis***	***Paenibacillus xylanexedens***	***Stenotrophomonas maltophilia***
Indole acetic acid (µg/l)	2030	730	1260	1490
Gibberellins (mg/l)	40.1	33.6	26.9	29.3
Polysaccharides (mg/l)	55.8	37.2	11.2	31.4

2. Optimization of PGPR Antagonistic Capabilities

Data presented in Table (2) indicate that while glucose supported the highest antagonistic impact against *Eschericia fergusonii* with inhibition zone of 29, 27 and 22 mm, for *Bacillus altitudinis*, *Brevibacillus brevis* and *Paenibacillus xylanexedens*, respectively, lactose was not that preferable, while glycerol was preferable to *Stenotrophomonas maltophilia* with inhibition zone of 30 mm comparing with 19 mm by using glucose. Nutritional factors considerably control the antagonistic effect. Differences in zone of inhibition among bacterial strains and among media depend on the composition of the medium (Kirtiwar et al., 2019)

Adding easily available carbon sources result in high antagonistic effects of bacteria (Meidute et al., 2008). Antimicrobial production impacted by the variant in carbon sources and in respect to the bacterial strain. Glucose increased the antimicrobial activity according to Gebreel et al. (2008) and Rana and Sahgal (2017) also strongly enhanced the antimicrobial activity for *Bacillus subtilis* and *Bacillus cereus* while glycerol increased the antimicrobial activity for *Bacillus circulans* and *Bacillus megaterium* (El-Banna, 2005).

Apart from the PGPR strain, high suppression of the pathogen expressed in 35 mm inhibition zone diameter with *Bacillus altitudinis* as attributed to L-glutamic acid as a nitrogen source, comparing with other nitrogen sources. For *Paenibacillus xylanexedens* and *Stenotrophomonas maltophilia*, the inhibition zones were 20 and 21 mm, respectively. Peptone and sodium nitrate with *Brevibacillus brevis* gave the highest suppression zone with the pathogen by 26 mm per each, comparing with other nitrogen sources. Ammonium nitrate was the inferior (11 mm, in average) among the tested nitrogen supplies. Krupodorova et al. (2019) mentioned that the highest antimicrobial activity was observed with ammonium nitrate or peptone as a carbon source. In previous study peptone and ammonium nitrate showed the

maximum antimicrobial activity for *B. stratosphericus* (Durairaj et al., 2017). Peptone also was recognized as nitrogen source that remarkably affected antibiotic production for *Streptomyces* (Oskay et al., 2011). Arasu et al. (2014) reported that optimum conditions for better antibacterial activity were at glucose as a carbon source, ammonium nitrate as a nitrogen source, pH 7 with incubation period of 96 h at 30°C incubation temperature.

Antimicrobial activity may not operate well under conditions such as low temperatures and (Omar et al., 2006). At incubation temperature of 30ºC, the suppression effect was the highest for *Bacillus altitudinis* and *Paenibacillus xylanexedens* by 29 and 19 mm inhibition zone, respectively. The majority of PGPR strains kept, to lower extent, their antagonistic capabilities when the incubation temperature rose to 35ºC, while the inhibition zones reached the highest with *Brevibacillus brevis* and *Stenotrophomonas maltophilia* by 30 and 29 mm, respectively. Increasing the temperature significantly reduces suppression activity (Islam et al., 2012; Yaseen et al., 2017 and Meena et al., 2020). Fengicin antibiotic produced by *B. subtilis* BBG208 strain, reaches the highest yield at 30ºC and pH 7 (Yaseen et al., 2017), Moreover, *B. subtilis* MS21 strain reaches the optimum antagonistic activity at 35ºC and pH 8 (Anjhana and Sasikala 2017).

As expected, all the tested PGPR strains exhibited better antagonistic potentials in culture media of pH 7.0 (Arumugam, 2017) where the inhibition areas were the widest (20 – 31 mm). All antagonists failed to antagonize the pathogen when grown in acidic or alkaline culture media. Antagonistic activity, might be released according to enzyme production, depends greatly on pH as a key mainly near neutral (from 5-7) (Shanmugaiah et al., 2008). *B. subtilis* bacteria can grow and synthesize antibiotics at pH values from 5 to 9 and in the temperature range from 25 to 37ºC (Chen et al., 2015). Additionally, with antagonistic activity against *Rhizoctonia solani* it was able to grow at temperatures from 15 to 37ºC and in pH from 5 to 9 (Mousivand et al., 2012) and at 28°C with pH 5-8 according to Jiménez-Delgadillo et al. (2018).

The inhibitory outcome fully depends on the cultivation conditions on the media and the incubation period regarding each other (Matevosyan et al., 2019), and the incubation period depends on the microbial strain (Balouiri et al., 2016).

The incubation period of 48 h seemed the most appropriate for the antagonists to express themselves as bio-agents, inhibition zone diameters were falling in the range 24-33 mm. Incubation time of 96 h at 30°C and pH of 7.0 was suitable for the optimal yield of antimicrobial compound (Kiran et al., 2018). The highest activity for *Bacillus coagulans* against Gram-positive, Gram-negative and yeasts was after 24h at 30°C and pH of 8.0.

Table (2). Antagonistic impacts of PGPR strains against *Escherichi fergusonii* as affected by cultivation conditions.

Isolates / Treatments	*Bacillus altitudinis*	*Brevibacillus brevis*	*Paenibacillus xylanexedens*	*Stenotrophomonas maltophilia*
Carbon source (1%, w/v)			**Inhibition zone (mm)**	
Glucose	29	27	22	19
Glycerol	13	22	17	30
Lactose	25	9	7	10
Mannitol	16	20	19	27
Sucrose	10	28	9	8
Nitrogen source (0.05%, w/v)			**Inhibition zone (mm)**	
Ammonium chloride	20	22	19	19
Ammonium nitrate	12	10	10	11
L-Glutamic acid	35	20	20	21
Peptone	29	26	17	19
Sodium nitrate	33	26	11	18
Incubation temperature			**Inhibition zone (mm)**	
15	0	0	0	0
20	8	0	0	0
25	25	20	16	20
30	29	27	19	23
35	21	30	14	29
40	0	0	0	11
50	0	0	0	0
Incubation period			**Inhibition zone (mm)**	
24	20	23	19	18
48	33	30	24	27
72	30	30	20	25
96	20	28	21	20
120	32	28	22	23
pH			**Inhibition zone (mm)**	
4.0	0	0	0	0
5.0	6	0	0	0
6.0	9	17	0	9
6.5	29	20	6	30
7.0	31	30	20	28
7.5	8	13	20	11
8.5	0	0	12	6
9.5	0	0	0	0
Salinity (NaCl, %)			**Inhibition zone (mm)**	
0.0	30	28	19	20
1.5	34	30	16	26
2.5	26	26	0	26
3.5	0	6	0	8
4.5	0	0	0	0
5.5	0	0	0	0
6.5	0	0	0	0

While both unsalted- or 1.5% NaCl-supplemented- culture media supported the highest antagonistic efficiency of the PGPR strains (16–34 mm), the salt concentrations above 3.5% dramatically reduced the bio-agent activities. Kiran et al. (2018) reported an antimicrobial activity in media supplemented with 2% NaCl with a zone of inhibition of 23.2 mm.

CONCLOUSION

The present study focused on the preferable cultivation conditions that support antibiosis capabilities for the tested PGPR antagonists. The bacterial strains were allowed to grow in culture media of different carbon, nitrogen sources and sodium chloride levels and incubated at various temperatures for prolonged incubation times, thereafter; their antagonistic activities against *E. fergusonii* were monitored. For the majority of PGPR, the highest pathogen suppressions were due to strains harvested from neutral nutrient media (pH, 7.0) enriched with glucose and L-glutamic acid with or without 1.5% NaCl and incubated at 30°C for 48 h.

Fertilizers are a determining practice in continuing crops growth and crucial yield. Even so using of chemical fertilizers continuously could infect the soil ecosystem and in the long run limit nutrient uptake by plant roots. So, biological fertilizers could be an alternative solution instead of chemical fertilizers to sustain crops cultivation. In this study, a total of four antagonistic rhizobacteria were isolated. The isolated bacteria were identified as *Bacillus altitudinis, Brevibacillus brevis, Paenibacillus xylanexedens and Stenotrophomonas maltophilia*. These bacteria were effective in producing antibacterial products and were able to produce IAA, GA and polysaccharides. In conclusion, these rhizobacterial isolates are effective to be not only as biocontrol agents to minimize the hazardous chemicals effect in disease management, but also effective as bio-fertilizers to improve plant growth due to their potent in plant growth-promotion.

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تأثير عوامل النمو على نشاط بعض بكتريا الريزوسفير المضاد لبعض الممرضات البكتيرية

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تمت دراسة نواتج التمثيل الحيوي الميكروبي لعزلات بكتيرية ذات كفاءة في إنتاج المضادات الحيوية بعد تعريفها بواسطة الخواص البيوكيميائية والتسلسل الجيني 16S rRNA من أجل تحديد الظروف المثلى لكل من تركيب بيئات النمو وظروف التنمية الملائمة، للحصول على أعلى إنتاجية من المضادات الحيوية. تمت دراسة مصادر كربون مختلفة (جلوكوز - جليسرول - لاكتوز- مانيتول - سكروز) ومصادر نيتروجين مختلفة (كلوريد أمونيوم - نترات أمونيوم - حمض الجلوتاميك – ببتون - نترات صوديوم) وكذلك درجات حموضة (٤.٠-٩.٥) ودرجات تحضين (١٥، ٢٠، ٢٥، ٣٠، ٣٥، ٤٠، ٥٠ درجة مئوية) ودرجات ملوحة (١.٥، ٢.٥، ٣.٥، ٤.٥، ٥.٥، ٦.٥ فى المئة) وأوقات تحضين (٢٤، ٤٨، ٧٢، ٩٦، ١٢٠ ساعة) وذلك بغرض دراسة تأثيرها على النشاط التضادي للسلالات محل الدراسة باستخدام تكنيك الانتشار على الأجار وتعيين حجم هالة التضاد. وقد اتضح وجود تأثير قوى لكل من تركيب البيئة وكذلك ظروف النمو على نشاط التضاد الميكروبي لعزلات بكتريا الريزوسفير المختبرة *Bacillus altitudinis*, *Brevibacillus brevis*, *Paenibacillus xylanexedens* *Stenotrophomonas maltophilia* ضد بكتريا *Eschericia fergusonii*. وتم ملاحظة أن أعلى نشاط تضادي للسلالات كان في البيئات المتعادلة الحموضة المحتوية على الجلوكوز كمصدر كربون وحمض الجلوتاميك كمصدر للنيتروجين في وجود أو عدم وجود كلوريك الصوديوم بتركيز ١.٥٪ وذلك لعد فترة تحضين ٤٨ ساعة على درجة حرارة ٣٠ درجة مئوية وكانت هالة التضاد قطرها ٣٠ ملليمتر.