Evaluation of some Plant Growth Promoting Rhizobacteria (PGPR) to Control *Pythium aphanidermatum* in Cucumber Plants

Ahmed M. Elazzazy[1*], Omar A. Almaghrabi [1], Tarek A. A. Moussa [1], Tamer S. Abdelmoneim[1,2]

[1]Biological Sciences Department, Faculty of Science (North Jeddah), King Abdulaziz University, P.O. Box 15758, Jeddah 21454, Saudi Arabia, [2]Suez Canal University, Faculty of Agriculture, Department of Agricultural Botany, P.O. Box 41522, Ismailia, Egypt.

ahmedazazy8@hotmail.com

Abstract: Twenty isolates of bacteria were successfully isolated from rhizosphere soil samples collected from different plant species growing at various locations in North Jeddah, Saudi Arabia to investigate their effects on *Cucumis sativus* L. cv. Marketmore growth and damping-off disease caused by *Pythium aphanidermatum*. The results of dual culture method showed that only three isolates out of twenty, which have a great antagonistic effect on the growth of *P. aphanidermatum* referring to inhibition index. The selected isolates were identified in two genera with three species as *Bacillus subtilis, B. amyloliquefaciens* and *Pseudomonas aeruginosa*, that causing inhibition in fungal growth by rate 38.1±3.8, 66±5.0 and 78.1±6.8%, respectively. Under greenhouse condition, the efficacy of different treatments with bacterial isolates and their possible combination were recorded a varied significant effect to suppress damping off symptoms caused by *P. aphanidermatum.* All treatments with the isolates of PGPR were recorded growth promoting effect in the absence of pathogenic fungus comparing with untreated plant. The best result in plant height, stem length, plant fresh and dry weight were recorded in treatment with *B. subtilis* (23.7±1.04 cm, 13.5±0.87 cm 0.62±0.02 g and 0.033±0.007 g), flowed by *P. aeruginosa* (19.3±1.15 cm, 9.00±1.0 cm, 0.61±0.07 g, 0.029±0.002 g) then *B. amyloliquefaciens* (17.0±1.30 cm, 8.97±0.90 cm, 0.53±0.01 g, 0.031±0.004 g). On contrast, all seeds were completely dead, when treated with *P. aphanidermatum* individually or in combination with PGPR isolates except two treatments, which used *B. amyloliquefaciens* and *P. aeruginosa* for coating seeds. Consequently, these two isolates have the potential not only to protect cucumber seedling against *P. aphanidermatum* infection but also to improve the plant growth parameters.

[Ahmed M. Elazzazy, Omar A. Almaghrabi, Tarek A. A. Moussa, Tamer S. Abdelmoneim**. Evaluation of some Plant Growth Promoting Rhizobacteria (PGPR) to Control *Pythium aphanidermatum* in Cucumber Plants.** *Life Sci J* 2012;9(4):3147- 3153] (ISSN:1097-8135). http://www.lifesciencesite.com. 461

Keywords: Damping off, PGPR, Cucumber plant, Biological control.

1. Introduction

Cucumber plants (*Cucumis sativus* L.) are one of the main greenhouse crops widely grown in Saudi Arabia. The total planting area for cucumber production decreased from 3636 hectares in 2006 to 3149 hectares in 2010, and production decreased from 242004 ton in 2006 to 220978 ton in 2010. Among all vegetable produced in greenhouse, cucumber production area decreased from 3.2% to 2.8% and yield decreased from 9.2% to 8.7%, respectively (Ministry of Agriculture, 2011).

One of the major factors inhibits growth and yield of cucumber is damping-off caused by *Pythium* species. In most cases Pre- and post-emergence damping-off in cucumber is caused by *Pythium aphanidermatum* and other *Pythium* spp. (Abbasi and Lazarovits, 2006). In major *Pythium* can result economic losses in both greenhouse and field production systems under favorable conditions for disease development (Georgakopoulos *et al.,* 2002; Abbasi and Lazarovits, 2006). The damage effects causing by *Pythium* spp. can increase in the association with some plant-parasitic nematode (*Meloidogyne incognita)*, which known as complex diseases that can cause highly significant losses to field and greenhouse grown cucumbers and other cucurbits (Zitter *et al.,* 1996; Koening *et al.*, 1999). They are ubiquitous in soil and in water, distributed worldwide, and with very diverse host ranges. *Pythium* species include some of the most important and destructive plant pathogens, causing losses of seeds, pre-emergence and post-emergence damping-off. In addition to causing rots of seedlings, roots, or basal stalks, decays of fruits and vegetables during cultivation, storage, transit or at the market and serious damages of a wide variety of crops in KSA (Yu and Ma, 1989; Al-Sheikh, 2010).

The part of problem can effectively be solved by the use of chemical fungicides. However the indiscriminate use of chemical fungicides is not recommended for the management of plant diseases because of their collateral adverse effects on the environment, along with negative effects on animal and human health. Moreover, their efficacy has been reduced by the appearance of microbial resistance (Sanders, 1984; Cook and Zhang, 1985) and their

detrimental effect on the biological nitrogen fixation by rhizobia (Altier and Pastorini, 1988).

Many studies are interest in biological control by beneficial microorganisms (Moussa, 2002; Kim *et al.*, 2008a, b, 2009; An *et al.*, 2010; Sang *et al.*, 2011) or composts (Sang *et al.*, 2010; Sang and Kim, 2011) has increased consistently as an alternative disease control to substitute for various hazardous chemical controls against airborne or soil borne plant pathogens (Lamour and Hausbeck, 2000; Kim *et al.*, 2008c; Kim and Kim, 2009). In this regard some rhizobacterial isolates were isolated from root surface or interior, which known as beneficial microorganisms and have been utilized as bio-control agents. These beneficial bacteria have the ability to colonize rhizosphere or plant roots and/or to produce secondary metabolites including antibiotics, extracellular enzymes, hydrogen cyanide (HCN), siderophores, and phytohormones (Kamilova *et al.*, 2005; Kim *et al.*, 2009).

The objectives of this study was established to isolate some bacteria with antagonistic activity against *Pythium aphanidermatum* for potential use as biocontrol agents, investigation of the effect of selected bacteria on *Pythium* damping-off control and finally, providing information valuable for use in integrated programs for damping-off management in KSA.

2. Material and Methods

2.1. Isolation and purification of bacteria

One gram of dry soil sample was added to 100 ml of nutrient broth medium (pH 6.6-7.0) in 125-ml Erlenmeyer flask. The mixture was shaken for 3 min and incubated for 6 hrs at 25±1ºC. Then loopfull's of the resulting suspension were streaked into plates of nutrient agar medium plates and allowed to grow. Colonies that were formed after incubation at 30ºC were selected and streaked again on N-agar medium to obtain pure cultures. All isolates were maintained on nutrient agar slants at 4ºC for the further experiments. Bacteria were purified through the single colony technique used for isolation and purification of the bacterial culture (Salle, 1954). The isolated bacteria were identified according to Bergey's Manual of Determinative Bacteriology (George, 2005) based on the characters such as morphology, physiology and nutritional, cultural characteristics and biochemical tests.

2.2. Agar plate-based fungal inhibition assays

Bacterial strains were tested for pathogen inhibition on potato dextrose agar (PDA). *Pythium aphanidermatum* was maintained on 1x PDA. For inhibition assays, a 5 mm-diameter agar plug of a 7-day-old culture of the pathogen was transferred to 1 cm from the edge of a plate of 1/4 x PDA and incubated at 23± 2 °C in darkness. After 24 hrs, 5 µl from an exponentially growing bacterial culture at optical density 600 of 0.1 was spotted 1 cm from the other edge of the 1/4 x PDA plate. The inhibition index was defined as:-

$$\textbf{Inhibition index} = [\boldsymbol{y} \div (\boldsymbol{x} + \boldsymbol{y})] * \mathbf{100}$$

Where "y" is the distance between the leading edge of the fungus and the edge of the bacterial colony, and "x" is distance between the leading edge of the fungus and the center of the agar plug (McSpadden and Weller, 2001). Assays were conducted three times, with each strain replicated twice in each assay.

2.3. Greenhouse experiment

2.3.1. Preparation of pathogenic inoculums:

An aggressive isolate of *Pythium aphanidermatum* was kindly supplied from Microbiology Lab., National Research Center (NRC), Egypt. The isolate was cultivated on autoclaved barley grains for 7 days at 21±2°C. The fungal inoculum was calculated based on the number of fungal propagules (FP) present in 1 g inoculum (1.3×10^7 g^{-1} inoculum).

2.3.2. Inoculation and experimental design:

Five seeds of *Cucumis sativus* L. cv. Marketmore were planted in plastic bags containing 500 g of sterilized soil (pH 6.7 the soil had 33.4 mg kg^{-1} extractable N, 6.2 mg kg^{-1} extractable P, and 44.6 mg kg^{-1} extractable K). Sixteen treatments with three replicates were used as follows: 1- Soil was infested with phytopathogenic fungus (*Pythium aphanidermatum)* before planting time by rate 1.3×10^7 FP g^{-1} soil. 2- Seeds of *Cucumis sativus* L. cv. Marketmore were coated by using 1 ml from pure culture for the different three isolate of PGPR (separately) by rate 3.6×10^6, 1.2×10^6 and 2.4×10^6 CFU ml^{-1} to *Bacillus subtilis*, *B. amyloliquefaciens* and *Pseudomonas aeruginosa*, respectively. 3- Seeds were treated with different possible combination between the three bacterial isolates (*B. subtilis*, *B. amyloliquefaciens* and *P. aeruginosa*) by the same rate. 4- Seeds were planted in the presence of pathogenic fungus and antagonistic bacteria as described in previous steps (2 and 3). 5-Seeds were planted free from any treatments including microorganisms (non-infested control).

Seed germination and seedlings were check daily to determine plant survival and recording any changes in seedling. At the end of the experiment (14 days) plants were removed from the soil then plant growth parameters were determined. As well as final

germination percent (FGP) (ISTA, 1993; 1999) was calculated based on the following equation:-

$$FGP = \frac{\text{Number of germinating seeds}}{Total\ Numbeer\ Of\ seeds} \times 100$$

Mean daily germination (MDG) was calculated according to the following equation (Moradi *et al.*, 2008):-

$$MDG = \frac{FGP}{d}$$

Where "d" are the days to the maximum of final germination.

The germination index (GI) was calculated as described in the Association of Official Seed Analysts (AOSA, 1983) by following formula:-

$$GI = \frac{No.of\ germinated\ seed}{Days\ of\ first\ count} + - - + \frac{No.of\ germinated\ seed}{Days\ of\ final\ count}$$

The infection index (II) was calculated as follows:-

$$II = \frac{No.of\ infected\ seed}{Days\ of\ first\ count} + - - + \frac{No.of\ infected\ seed}{Days\ of\ final\ count}$$

3. Statistical analyses

One-way ANOVA and Duncan multiple range test were used to evaluate the significant difference in the concentration of different study sites. A probability at level of 0.05 or less was considered significant (Bailey, 1981). Standard errors were also estimated.

3. Results and Discussion:

A total of 20 isolates for plant growth promoting rhizobacteria (PGPR) were isolated on N-agar medium from soil samples, which collected from various locations in North Jeddah, KSA. Under *In vitro* conditions isolates B_1, B_2 and B_3 were showed highly significant reduce of fungal mycelium growth to *Pythium aphanidermatum* (38.1±3.8%, 66.0±5.0% and 78.1±6.8%, respectively) on PDA plates in a triplicate assay (Table 1). These results are in agreement with many previous studies reported that the antagonistic activity of some isolates of PGPR against different damping-off disease caused on various crops (Baker and Paulitz, 1996, Anjaiah *et al.*, 1998, Harris and Adkins, 1999, Tambong and Hofte, 2001, Perneel *et al.*, 2007, 2008). The inhibitory effects were more prominent on PDA than on NA medium. The nutrient constituent of the media plays a significant role in influencing the production of a particular antifungal metabolite (Hebbar *et al.*, 1992b) by the antagonistic rhizobacteria. The differences in the inhibitory effect on the fungal pathogens might be due to the nutritional differences of the two media. Similar observations were also reported earlier with *Pseudomonas cepacea* (Hebbar *et al.*, 1992a). The selected isolates were identified in two genera with three species as *Bacillus subtilis*, *B. amyloliquefaciens* and *Pseudomonas aeruginosa* (B_1, B_2 and B_3, respectively).

Table 1. The influence of 20 isolates of PGPR against aggressive isolate from *Pythium aphanidermatum in vitro*

Bacterial isolate	Inhibition index* (%)
B_1 ✖	38.1± 3.8
B_2 ✖	66.0± 5.0
B_3 ✖	78.1±6.8
B_4	25.8 ±3.3
B_5	21.0 ±2.5
B_6	15.6 ±1.3
B_7	3.50 ±0.6
B_8	13.5 ± 1.9
B_9	5.00 ± 1.6
B_{10}	5.10 ± 1.0
B_{11}	15.5 ±2.2
B_{12}	12.5 ±2.0
B_{13}	0.1 ± 0.1
B_{14}	7.00 ±0.7
B_{15}	5.00 ±0.7
B_{16}	0.0 ± 0.0
B_{17}	15.5 ±2.2
B_{18}	4.00 ±2.2
B_{19}	20.0 ±2.8
B_{20}	0.0 ± 0.0

The data are the mean of three replicates ± SD, *Inhibition index defined as [y/(x+y)](100), where x is the distance from the center of the plug to the leading edge of the fungus and y is the distance from the edge of the bacterial colony to the growing edge of the fungus, ✖ Selected isolates for *in vivo* experiment.

Data presented in Table (2) showed that the efficacy of the three isolated species from PGPR on growth parameters of *Cucumis sativus* L. cv. Marketmore in the presence or absence of aggressive isolate for pathogenic fungus *P. aphanidermatum* under greenhouse condition. The highly values to plant growth parameters were recorded in all treatments in the absent of pathogenic fungus with the presence of *Bacillus subtilis* alone or in combination with the other two isolates. The best result in plant height, stem length, plant fresh and dry weight were recorded in treatment with *B. subtilis* (23.7±1.04 cm, 13.5±0.87 cm 0.62±0.02 g and 0.033±0.007 g), flowed by *P. aeruginosa* (19.3±1.15 cm, 9.00±1.0 cm, 0.61±0.07 g, 0.029±0.002 g), then *B. amyloliquefaciens* (17.0±1.30 cm, 8.97±0.90 cm, 0.53±0.01 g, 0.031±0.004 g). These results are in agreement with (Kamilova *et al.*, 2005; Kim *et al.*, 2009). While in the presence of pathogenic isolate of *P. aphanidermatum* caused completely death in all treated seeds due to pre-emergence damping-off.

Similar results recorded by Lee *et al.* (2010) who recorded that, *P. aphanidermatum* was a very aggressive strain caused pre-and post emergence damping-off in cucumber plants.

On the other hand, two isolates of PGPR (*P. aeruginosa* and *B. amyloliquefaciens*) have a good antagonistic effect to inhibit or suppress the effect of Pythium diseases when using separately.

These results were in agreement with some previous studies indicated that *P. aeruginosa* produces different types of antibiotic that play a major role in suppression of *Pythium* (Tambong and Hofte, 2001; Perneel *et al.*, 2008).

In addition to control of pathogenic fungus by using isolate of *Pseudomonas* has been reported to be due to competition for iron, antibiosis and induced systemic resistance in the host (Pieterse *et al.*, 2001; Berg *et al.*, 2002). However, further studies will be required to investigate factors involved in suppression of *P. aphanidermatum* induced damping-off using *P. aeruginosa*. Also many studies have shown that cucumber growth was affected by *B. amyloliquefaciens* in the presence of damping off causal fungi (Baker and Paulitz, 1996; Harris and Adkins, 1999).

The effects of bacterial combination on suppression effect of damping off disease, that due to the antagonism between the bacterial strains may be attributed to the competition, which occurs between the organisms, require the same nutrients by one reduce the amount available to the other. In addition to the toxins or antibiotics secreted in the growth area as well as the production of antifungal volatiles (Lefiert *et al.*, 1995; Saleh, 1997; Walker *et al.*, 1998; Abo-Elnaga, 2006).

Data illustrated in Figure 1, showed that the using of *B. subtilis* alone or mixed with the other isolates (*P. aeruginosa* and *B. amyloliquefaciens*) was significant increased in the values of final germination percent (FGP%) to cucumber cultivar comparing with infested treatments, as well as germination index (GI), on the other hand, infection index (II) were significantly increased by decreasing in values of suppression effect with related to infested control. Chemical control of root diseases is often inconsistent and is a target for public concern due to possible environmental consequences; however, selected biocontrol agents may be potentially effective in suppressing disease in the field (Weller and Cook, 1983). Cropping systems and soil properties influence both detrimental and beneficial microorganisms in the rhizosphere, which subsequently impact root health, plant vigor, and crop yield (Rovira *et al.*, 1990; Hornby and Bateman, 1997).

Table 2. The efficacy of three isolated species from PGPR on growth parameters of *Cucumis sativus L. cv. Marketmore* In the presence or absence of pathogenic Pythium aphanidermatum under greenhouse condition

Plant treaded with	Plant growth parameters					
	Length (cm)			Weight (g)		Chlorophyll content (Unit)
	Plant	Stem	Root	Fresh	Dry	
Untreated	17.5±0.8	7.17±0.72	10.33±1.59	0.25±0.06	0.027±0.005	12.45±3.38
Pythium aphanidermatum (Pa)	P*	P	P	P	P	P
Bacillus subtilis (*Bs*)	23.7±1.04*	13.5±0.87*	10.37±0.29	0.62±0.02*	0.033±0.007*	16.05±1.5*
Bacillus amyloliquefaciens (*Ba*)	17.0±1.30	8.97±0.90	8.030±2.00	0.53±0.01*	0.029±0.004*	12.43±0.50
Pseduomonas aeruginosa (*Psa*)	19.3±1.15*	9.00±1.00	10.13±2.08	0.61±0.07*	0.031±0.002	16.00±5.15*
(Bs) + (Ba)	22.0±5.20*	10.5±1.32*	11.50±5.70	0.65±0.01*	0.037±0.003	16.03±3.03*
(Bs) + (Psa)	21.83±0.7*	9.00±2.00	12.8±2.70*	0.59±0.03*	0.029±0.001	12.6±1.90
(Ba) + (Psa)	15.7±0.20	10.0±0.87	5.67±0.58	0.19±0.03	0.020±0.002	11.27±0.8
(Bs) + (Ba)+(Psa)	11.83±1.6	5.00±1.00	6.83±1.76	0.19±0.03	0.022±0.002	5.6±2.030
(Bs) + (Pa)	P	P	P	P	P	P
(Ba) + (Pa)	15.0±0.21	6.00±0.90	9.00±0.16	0.18±0.32	0.22±0.010	8.33±0.13
(Psa) + (Pa)	12.33±0.6	5.67±0.58	6.67±0.58	0.17±0.03	0.17±0.010	5.70±0.13
(Bs) + (Ba)+(Pa)	P	P	P	P	P	P
(Bs) + (Psa)+(Pa)	P	P	P	P	P	P
(Ba) + (Psa)+(Pa)	P	P	P	P	P	P
(Bs) + (Ba)+(Psa)+(Pa)	P	P	P	P	P	P

Mean of three replicates± SD, * significant at level 5%, *P: Plants can't survival under fungus infection rate (1.3×10^7 propagules g^{-1} soil).

Strains of rhizobacteria with ability to reduce severity of root diseases of cereal crops have been selected for field application to increase crop productivity (Lemanceau and Alabouvette, 1993; Hornby and Bateman, 1997). However, performance of selected rhizobacteria introduced into some field soils for disease suppression has been very inconsistent (Thomashow and Weller, 1996; Moussa *et al.*, 2012).

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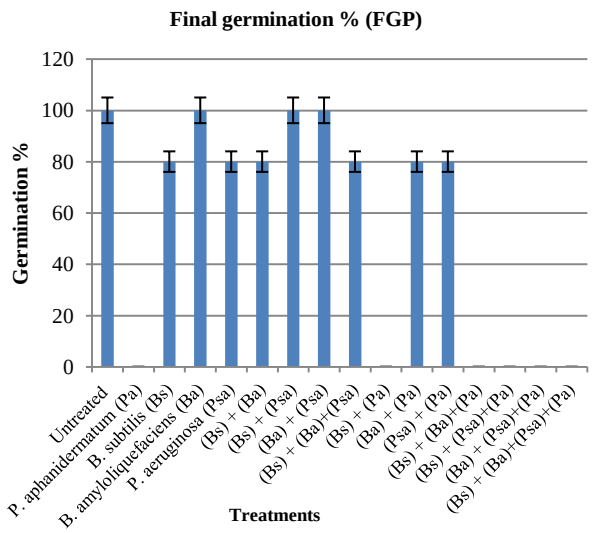

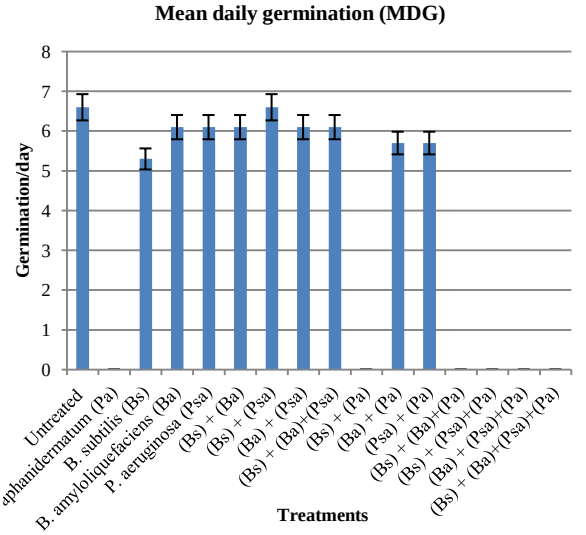

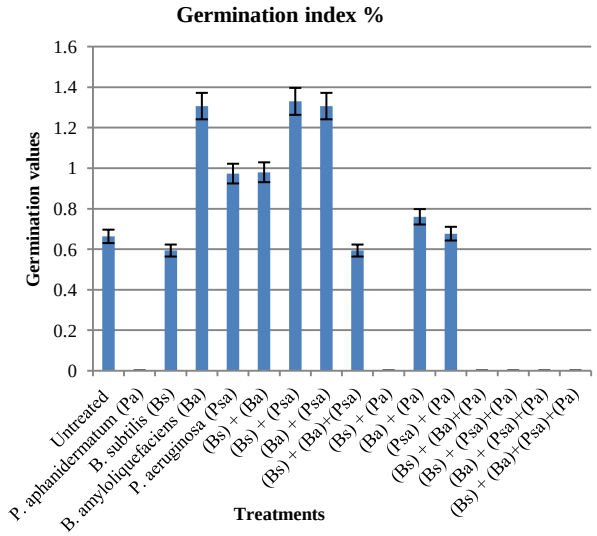

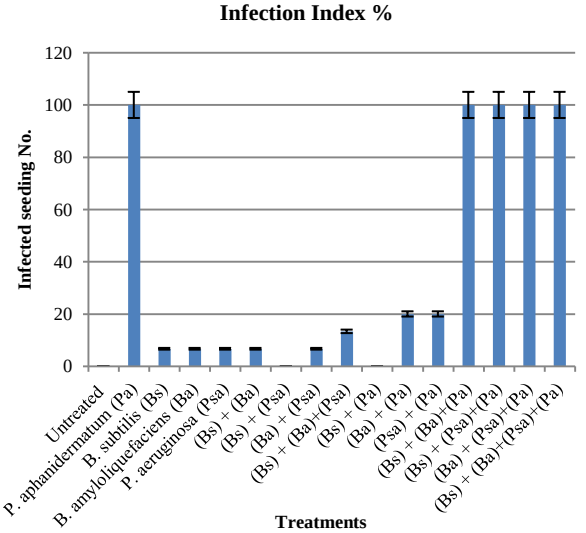

4. Conclusion

We conclude that, the selected isolates *B. amyloliquefaciens* and *Pseudomonas aeruginosa* significantly reduced the incidence of damping-off disease of cucumber caused by *Pythium aphanidermatum*. Fluorescent pigment and antifungal antibiotics (or metabolites) of *Pseudomonas aeruginosa* and antifungal antibiotics of both bacilli give successful root colonization of the biocontrol agents might be involved in biological suppression of the pathogens; and these strains have potential not only to protect cucumber seedling from *P. aphanidermatum* infection but also to improve the plant growth parameters

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11/11/2012