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Promotion of Cotton Seedlings Growth Characteristics by Development and use of New Bioformulations

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Abstract: This study was carried out at the Department of Plant Pathology of the Iranian Research Institute of Plant Protection during the period from January 2009 to December 2009 to develop some new bioformulations and evaluate their efficacy in promoting cotton seedlings growth characteristics. The interest in the use of biological approaches to replace chemical agents in fertilizing soils or improve plant resistance against phytopathogens is at present in continuous growth. In this regard, the use of Plant Growth Promoting Rhizobacteria (PGPR) has a potential role in developing sustainable systems for crop production. In search of efficient PGPR strains with multiple activities, we prepared eight bioformulations using two isolates of *Pseudomonas fluorescens* Q_{18} (B_1) and CKK-3 (B_2) which were isolated from rhizospheric soil and cotton roots in Varamin's cotton fields. Formulations included a talc-based powder and bentonite-based powder as inorganic carriers and peat and rice bran as organic carriers for increasing stability in interaction between associated PGPR and cotton plants. The results of a greenhouse experiment 60 days after sowing indicated that efficacy of BENT-B_1, TAL-B_1 and PT-B_1 treatments were 1.38, 1.35 and 1.27 fold, BENT-B_1, PT-B_1 and TAL-B_1 treatments were 1.42, 1.41 and 1.28 fold, RB-B_2, TAL-B_1 and RB-B_1 treatments were 3.44, 3.37 and 2.90 fold and TAL-B_1, RB-B2 and RB-B_1 treatments were 2.60, 2.50 and 2.10, respectively on promoting seedling height, root length, seedling dry weight and root dry weight more effective than the control treatment.

Key words: PGPR, *Pseudomonas fluorescens*, organic carrier, mineral carrier

INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and or indirectly. In last few decades a large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have reported to enhance plant growth (Glick, 1995; Kloepper *et al*., 1989). The direct promotion by PGPR entails either providing the plant with a plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients like Nitrogen (N) or Phosphorus (P) from the environment.

The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effect of one or more phytopathogenic micro-organisms. The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (1) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene (Glick, 1995; Beneduzi *et al*., 2008), (2) asymbiotic N_2 fixation (Boddey and Dobereiner, 1995), (3) antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982), antibiotics (Shanahan *et al*., 1992) and cyanide (Flaishman *et al*., 1996) and (4) solubilization of mineral phosphates and other nutrients (Freitas *et al*., 1997). Most popular bacteria studied and exploited as biocontrol agent includes the species of *Pseudomonas fluorescent* and *Bacillus*. Some PGPR may promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation or nodule occupancy (Fuhrmann and Wollum, 1989). However, role of cyanide production is contradictory as it may be associated with deleterious as

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well as beneficial rhizobacteria (Bakker and Schippers, 1987; Alstrom and Burns, 1989). In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competentable to survive and colonize in the rhizospheric soil (Cattelan *et al*., 1999). The PGPR concept has been documented by the isolation of many bacterial strains that fulfill at least two of the following criteria: aggressive colonization, plant growth stimulation and biocontrol (Haas and Defago, 2005).

Spore-forming bacteria such as *Bacillus* species are among the major types of soil bacteria. Common physiological traits important to their survival include production of a multilayered cell wall structure, formation of stress-resistant endospores and secretion of peptide antibiotics, peptide signal molecules and extracellular enzymes (Gardener, 2004). Quantitative and qualitative variations in these traits allow for these bacteria to inhabit diverse niches in agro-ecosystems. Their microscopic size and omnipresence in soil facilitate their colonization of plants and animals (Beneduzi *et al*., 2008). Stimulation and enhancement of root growth is one of the several methods of plant growth promotion by a group of bacteria commonly known as PGPR (Plant Growth Promoting Rhizobacteria). The PGPR has a close association with plant roots and can enhance the growth of many plants (Molla *et al*., 2001).

Cotton is an important cash crop which is being cultivated in many countries around the world including Iran (Naraghi *et al*., 2007). Among the biocontrol agents, Plant Growth Promoting Rhizobacteria (PGPR) viz., *Pseudomonas* sp. and *Bacillus* sp. have shown activity in suppressing the fungal infection and promoting growth characteristics (Chen *et al*., 2000). When PGPR are mixed with some other strains, or other bacteria or fungal antagonists the biocontrol efficacy is increased (Duffy *et al*., 1996). Thus, the objective of this study was to investigate of new bioformulations efficacy in promoting growth characteristics of cotton seedlings.

MATERIALS AND METHODS

Materials: Chemicals, microbial growth media and ingredient used for the preparation of various formulations were of laboratory chemical-reagent grade and were purchased from Tehran's chemical market, Iran. Media used in this study included Nutrient Agar (N.A) and Potato Dextrose Agar (PDA). Cotton (*Gossypium hirsutum* L.) seeds were provided by Iranian Research Institute of Plant Protection, Tehran, Iran. This study was conducted during the period from January 2009 to December 2009.

Microbial cultures: The bacterium, *P. fluorescens* Q_{18} and CKK-3 isolates, previously tested for their plant growth promoting traits such as production of Indole Acetic Acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN), siderophore, phosphate solubilization and antifungal activity, was obtained from the Microbial Culture Collection, Beneficial Microorganisms Research Laboratory, Iranian Research Institute of Plant Protection, Tehran, Iran. These bacterial isolates exhibited a high level of resistance to ampicillin and erythromycin, which was not observed in many native *P. fluorescens* isolates. The bacterial culture was maintained on Nutrient Agar (NA) medium. These isolates were sub-cultured once a month and maintained until the end of the experiment.

Development of formulations

Preparation of mineral and organic carriers: The two powdered minerals of talc (TAL) and bentonite (BENT) and two powdered organic compounds of peat (PT) and Rice Bran (RB) were chosen as carriers in this study. The carrier materials were steam sterilized at 140 kPa for 30 min and dried aseptically in glass trays for 12 h at 50°C before using.

Preparation of bacterial suspension: The *P. fluorescens* cells were harvested and centrifuged at 6000 rpm for 15 min and resuspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu mL^{-1} (OD_{620} = 0.3) and used as bacterial inoculum (Thompson 1996). These isolates were kept at -80°C in 44% glycerol and cells from stocks were first grown on KB medium. The inoculum was produced by transferring one loopful from that culture to 100 mL of KB broth in a 250 mL Erlenmeyer flask and incubating at room temperature (28±2°C) on a shaker at 150 rpm for 48 h.

Development of TAL-based, BENT-based, PT-based and RB-based formulations of *Pseudomonas* isolates: One loopful of individual *Pseudomonas* isolates was inoculated into KB broth and incubated on a shaker incubator at 150 rpm for 48 h at room temperature (28±2°C). After 48 h of incubation, the broth containing 9×10^8 cfu mL^{-1} was used for the preparation of TAL based, BENT-based, PT-based and RB-based formulations. To 400 mL bacterial suspension, a mixture of 1 kg of a purified TAL, BENT, PT or RB powder, 15 g calcium carbonate (adjusted to neutral pH) and 10 g carboxymethyl cellulose (CMC adhesive) was prepared under sterile conditions, following the method described by Vidhysekaran and Muthuamilan (1995). The product

Table 1: Description of different bioformulations developed in the study

Formulations/Treatments	Ingredients/Method
BENT-B_1	Suspension of *P. fluorescens* strain Q18 (400 mL) containing 9×108 cfu mL^{-1} mixed with fine grade bentonite (1 kg) and CMC (10 g)
BENT-B_2	Suspension of *P. fluorescens* strain CKK-3 (400 mL) containing 9×108 cfu mL^{-1} mixed with fine grade bentonite (1 kg) and CMC (10 g)
TAL-B_1	Suspension of *P. fluorescens* strain Q18 (400 mL) containing 9×108 cfu mL^{-1} mixed with fine grade talc (1 kg) and CMC (10 g)
TAL-B_2	Suspension of *P. fluorescens* strain CKK-3 (400 mL) containing 9×108 cfu mL^{-1} mixed with fine grade talc (1 kg) and CMC (10 g)
PT-B_1	Suspension of *P. fluorescens* strain Q18 (400 mL) containing 9×108 cfu mL^{-1} mixed with peat powder (1 kg) and CMC (10 g)
PT-B_2	Suspension of *P. fluorescens* strain CKK-3 (400 mL) containing 9×108 cfu mL^{-1} mixed with peat powder (1 kg) and CMC (10 g)
RB-B_1	Suspension of *P. fluorescens* strain Q18 (400 mL) containing 9×108 cfu mL^{-1} mixed with rice bran powder (1 kg) and CMC (10 g)
RB-B_2	Suspension of *P. fluorescens* strain CKK-3 (400 mL) containing 9×108 cfu mL^{-1} mixed with rice bran powder (1 kg) and CMC (10 g)

was shade dried to reduce the moisture content (less than 20%) and then packed in polypropylene bags and sealed.

Efficacy of formulations on the cotton seedling's growth characteristics under greenhouse conditions

Seed treatment/coating: For seed treatment, the seeds were initially surface sterilized with 1% sodium hypochlorite and soaked in double volume of sterile distilled water containing above mentioned formulations (10 g kg^{-1} of seed). After 12 h, the bacterial suspension was drained off and the seeds were dried under shade for 30 min and sown (Vidhyaskaran *et al.*, 1997).

Greenhouse studies: Various formulations were assessed for their efficacy on the seedling and root growth of cotton in greenhouse conditions. A pot culture study was undertaken with the following treatments by using Completely Randomized Design (CRD) with four replications. The formulations are shown in Table 1. Soil collected from a Varamin's cotton fields in Tehran province of Iran was air-dried, homogenized using a revolving jar mill and sterilized using a steam sterilizer for 3 h at 85°C. Pots (20 cm diameter) were filled with soil (3.5 kg). Twenty treated cotton seeds with *P. fluorescens* formulations were sown (depth 2 cm; spacing 2×3 cm) in each pot. Control (soil + untreated seeds) was also included.

Determination of cotton seedlings growth characteristics: Sixty Days after Sowing (DAS), the seedlings height, seedlings dry weight, root length and root dry weight were determined according to the procedures described by Newman (1966), Tennant (1975), Molla *et al.* (2001) and Cassan *et al.* (2009).

Statistical analysis: Experiment was performed in 9 treatments each with 4 replications. Analysis of variance and comparison means were done separately by the SPSS statistical package, 17 evaluation version. Data were tested by Duncan's Multiple Range Test (DMRT) (Table 3).

RESULTS AND DISCUSSION

From Table 2 it is evident that 60 days after sowing, average of cotton's seedlings length for BENT-B_1 and TAL-B_1 formulations were 1.37 and 1.35 fold as compared to the control. In this period, the average of seedlings roots length for BENT-B_1 and PT-B_1 formulations were 1.42 and 1.41 fold as compared to the control. Also, average of seedlings dry weight for RB-B_2 and TAL-B_1 were 3.44 and 3.37 fold as compared to the control. Finally, average of seedlings roots dry weight for TAL-B_1 and RB-B_2 formulations were 2.60 and 2.50 fold as compared to the control. Statistical analysis is shown in Table 3. From Table 3 in each group of data different letters show a statistically significant difference at the $p = 0.05$ level. Thus, results indicate that efficacy of BENT-B_1, TAL-B_1, PT-B_1 and RB-B_2 formulations on the cotton seedling and root growth characteristics were more effective than the control treatment.

The overall results of the study showed that most bioformulations promoted growth characteristics of the cotton seedlings compared to the control treatment. This is probably because the new bioformulations may play effective roles in increasing and establishment and durability of antagonistic microorganisms in soil and possibly produce antibiotics, siderophores, hydrolytic enzymes, phytohormones and/or other volatile extra-cellular metabolites.

Apparently the formulation of *P. fluorescens* strain Q_{18} (B_1) were more effective than that strain CKK-3 (B_2). This could be due to the differences in biochemical and genetic characteristics of this isolates.

In previous studies, Molla *et al.* (2001) evaluated the potential enhancement of root growth and nodulation in vegetable soybean (AGS190) with application of *Azospirillum brasilense* (Sp7) and *A. lipoferum* (CCM3863) co-inoculated with two *Bradyrhizobium japonicum* strains (TAL102 and UPMR48). They observed significant root growth stimulation and nodulation in *Azospirillum* as well as during its co-inoculation with *Bradyrhizobium*. Nodule formation is linked with the initiation of new roots; nodules were

Table 2: Assessment of efficacy of different treatments on cotton seedling growth characteristics 60 days after sowing (compared to untreated control)

Treatments	Seedling height (cm)	Root length (cm)	Seedling dry weight (g)	Root dry weight (g)
Control	39.90[a]	12.70[a]	0.97[a]	0.10
BENT-B_1	54.90	18.07	2.40	0.19
BENT-B_2	37.70	12.41	1.70	0.17
TAL-B_1	53.80	16.22	3.27	0.26
TAL-B_2	47.20	15.91	2.59	0.20
PT-B_1	50.60	17.95	2.57	0.18
PT-B_2	48.90	15.31	2.56	0.18
RB-B_1	44.50	14.56	2.81	0.21
RB-B_2	50.40	14.26	3.34	0.25

[a]The average of length/weigh in 4 replicates

Table 3: Statistical grouping of different treatments in their efficacy on cotton seedlings growth characteristics 60 days after sowing in the greenhouse according to Duncan multiple range test

Treatments	Average of seedling height	Average of root length	Average of weight seedling dry	Average of root dry weight
Control	BCD	B	B	B
BENT-B_1	A	A	AB	AB
BENT-B_2	CD	B	AB	AB
TAL-B_1	A	AB	A	A
TAL-B_2	ABC	AB	AB	AB
PT-B_1	AB	A	AB	AB
PT-B_2	ABC	AB	AB	AB
RB-B_1	ABC	AB	AB	AB
RB-B_2	AB	AB	A	A

LSD (p = 0.05)

almost absent even in *Bradyrhizobium* inoculated plant due to the absence of new roots development in clipped rooted seedlings. Total root length, root number, specific root length, root dry matter, root hair development and shoot dry matter were significantly increased by *Azospirillum* alone and its co-inoculum. Co-inoculated plants significantly influenced the number of nodules and its fresh weight. *Azospirillum brasilense* seemed to perform better in root growth and nodule development compared to *A. lipoferum*.

Bharathi *et al.* (2004) evaluated the efficacy of 13 plant growth promoting rhizobacterial strains against chilli fruit rot and dieback incited by *Colletotrichum capsici*. They observed among these formulations, *P. fluorescens* (pf_1) and *B. subtilis* to be effective in increasing the seed germination and seedling vigor. They also found that the PGPR mixed bioformulation (pf_1+*B. subtilis*+neem+chitin) was to be the best for reducing the fruit rot incidence, apart from increasing plant growth and yield parameters under both greenhouse and field conditions.

Kamrul Islam *et al.* (2006) evaluated the effect of biofertilizer (Bradyrhizobium) and plant growth regulators (GA_3 and IAA) on growth of summer mungbean. The experiment was laid out by RCBD with three replications and two factors (variety and treatment). There were altogether 12 treatment combinations. Most of the growth parameters such as number of branches plant, number of leaves plant, number of effective nodules plant, number of non-effective nodules plant, root dry weight plant, nodule dry weight plant was the height due to the application of biofertilizer. On the other hand, plant height, leave dry weight plant, shoot dry weight plant and total dry weight plant was the height due to the application of plant growth regulators. However, biofertilizer and plant growth regulators showed statistically identical performance on Crop Growth Rate (CGR) and Relative Growth Rate (RGR).

Abdul jaleel *et al.* (2007) surveyed the effect of PGPR including *Pseudomonas fluorescens* on growth parameters and the production of ajmalicin in *Catharanthus roseus* under drought stress. The plants under pot culture were subjected to 10, 15 and 20 Days Interval Drought (DID) stress and drought stress with *Pseudomonas fluorescens* at 1 mg L^{-1} and 1 mg L^{-1} *Pseudomonas fluorescens* alone from 30 Days after Planting (DAP) and regular irrigation was kept as control. The plants were uprooted on 41 DAS (10 DID), 46 DAS (15 DID) and 51 DAS (20 DID). Drought stress decreased the growth parameters and increased the ajmalicine content, but the treatment with *Pseudomonas fluorescens* enhanced the growth parameters under drought stress and partially ameliorated the drought induced growth inhibition by increasing the fresh and dry weights significantly. The ajmalicine content was again increased due to *Pseudomonas fluorescens* treatment to the drought stressed plants. From the results of this investigation, it can be concluded that, the seedling treatments of native PGPRs can be used as a good tool in the enhancement of biomass yield and alkaloid contents in medicinal plants, as it provides an eco-friendly approach and can be used as an agent in water deficit stress amelioration.

In another study, Cassan *et al.* (2009) tested the *Azospirillum brasilense* strain Az39 and *Brayrhizobium japonicum* strain E109 were previously shown to produce Indole 3-Acetic Acid (IAA), Gibberellic Acid (GA3) and Zeatin (Z) for early growth promotion in inoculated corn and soybean seedlings. They observed Az39 and E109, singly or in combination, showed the capacity to promote seed germination, nodule formation and early development of corn and soybean seedlings. Both strains were able to excrete IAA, GA3 and Z into the culture medium, at a concentration sufficient to produce morphological and physiological changes in young seed tissues.

Results of the above-mentioned studies clearly indicate that development of stable formulations of antagonistic bacteria is of great importance and is a promising approach to a sustainable agriculture. The results of present study in the development and formulation of PGPR bacteria may have practical application in biological promotion of plant growth

characteristics which can potentially replace the use of chemical fertilizers. The use and application of such bioformulations in the fields can result in the reduction of application of harmful chemicals, protect the environment and biological resources and can be an important component of Integrated Pest Management (IPM) that can help the growers to achieve a sustainable agriculture.

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