Development of *Pseudomonas fluorescens* and *Bacillus coagulans* based bioformulations using organic and inorganic carriers and evaluation of their influence on growth parameters of sugar beet

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ABSTRACT

Use of plant growth promoting rhizobacteria (PGPR) can play important roles in developing sustainable systems for crop production. In search for efficient PGPR strains with multiple activities, we prepared eight bioformulations (BF) using two isolates of *Pseudomonas fluorescens* (B_1) and *Bacillus coagulans* (B_2) which were isolated from rhizospheric soil and plant roots in Iranian sugar beet fields. Carriers included talc and bentonite powders as inorganic carriers and peat and rice bran as organic carriers for increasing stability in interaction between associated PGPR and sugar beet plants. The efficacy of prepared bioformulations were then evaluated on promoting sugar beet seedlings growth characteristics including seedling height, seedling dry weight, seedling dry weight, root length and root weight, 60 days after sowing. Results indicated that above-mentioned growth characteristics except root length were significantly increased by all test BF but with different ratio. However, in the case of root length, 5 out of 8 BF (Peat-b1, Talc-b1, Talc-b2, R.B.-b1 and R.B.-b2) showed significant effectiveness in increasing seedling root length. The overall results of this study show that it may be possible to use PGPR based BF for promoting the health and growth of sugar beet.

Key words: PGPR, sugar beet, organic carriers, inorganic carriers.

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 and promote the quantity and quality of the plant growth characteristics. In the last few decades, a large group of bacteria including species of Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthobacter, Burkholderia, Bacillus and Serratia has been reported to increase and promote plant growth characteristics directly or indirectly (Glick, 1995; Kloepper *et al.*, 1989). The direct promotion by PGPR includes either providing the plant with plant growth enhancing substances that are produced by the bacterium or facilitating the uptake of certain plant nutrients like nitrogen (N) or phosphorus (P) from the environment.

The indirect effects on plant growth occurs when PGPR decrease or prevent the harmful effect of one or more plant pathogenic micro-organisms. The exact mechanisms and mode of actions of PGPRs are not fully understood, but are thought to include the following: 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- symbiotic N_2 fixation (Molla *et al.*, 2001), 3- antagonism against phytopathogenic microorganisms by production of siderophores antibiotics (Shanahan *et al.*, 1992) and cyanide (Flaishman *et al.*, 1996), and 4-solubiliz ation of mineral phosphates and other nutrients (De Freitas *et al.*, 1997).

The most important and effective bacteria studied and exploited as biocontrol agents include the species of *Pseudomonas* and *Bacillus*. Some PGPR may promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation or nodule occupancy (Fuhrmann and Wollum, 1989). However, the role of cyanide production is contradictory as it may be associated with deleterious as well as beneficial rhizobacteria (Alstrom and Burns, 1989; Bakker and Schippers, 1987). In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able 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: colonization, plant growth stimulation, and biocontrol.

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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 agroecosystems. Their microscopic size and omnipresence in soil facilitate the 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. The PGPR has a close association with plant roots and can enhance the growth of many plants (Molla *et al.*, 2001).

Sugar beet is an important cash crop which is being cultivated in many countries around the world including Iran (Shah-Smith and Burns, 1997; Collins and Jacobson, 2003; Shahraki *et al.*, 2008, 2009). Among the biocontrol agents, bacterial antagonists including *Pseudomonas* spp. and *Bacillus* spp. have shown activity in suppressing the fungal infection and promoting its growth characteristics (Chen *et al.*, 2000). Since we have previously tested bacterial isolates used in this study on sugar beet as suspension and have achieved promising results (Shahraki *et al.*, 2008, 2009) and in order to make the use of PGPR easier and to improve their stability and durability in the rhizosphere, we decided to conduct this study. According to the results of previous studies (Shah-Smith and Burns, 1997; Bharathi *et al.*, 2004) when PGPR are formulated using inorganic or organic carriers, their stability and durability are increased. In addition, their application particularly as seed treatment becomes easier and more practical. Thus, the objectives of this study were to develop and prepare some new bioformulations and evaluate their efficacy in possible promotion of sugar beet seedlings growth characteristics.

MATERIALS AND METHODS

Materials

Chemicals, microbial growth media and ingredients used for the preparation of various bioformulations were purchased from Tehran chemical market, Iran. Media used in this study included Nutrient Agar (NA) and Potato Dextrose Agar (PDA). Sugar beet, *Beta vulgaris* L. seeds were provided by Iranian Research Institute of Sugar beet.

Microbial Cultures

Two bacterial strains of *Pseudomonas fluorescens* (B1) and *Bacillus coagulans* (B2) previously tested for their plant growth promoting traits such as production of ammonia (NH_3), hydrogen cyanide (HCN) siderophore, phosphate solubilization and antifungal activity, were obtained from the Microbial Culture Collection, Beneficial Microorganisms Research Laboratory, Iranian Research Institute of Plant Protection, Tehran, Iran. The bacterial culture was maintained on Nutrient Agar (NA) medium (Difco Company). These isolates were sub-cultured once a month and maintained until the end of the experiment (Shahraki *et al.*, 2009).

Preparation of inorganic and organic carriers

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 use. It is notable that all carriers were obtained from Tehran chemical company and market, Tehran, Iran.

Preparation of bacterial suspension

Bacterial 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 10^9 cfu ml^{-1} and was 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 and preparation of bioformulations

One loop of individual bacterial 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 10^9 cfu ml^{-1} was used for the preparation of TAL based, BENT-based, PT-based and RB-based formulations. To 40 ml bacterial suspension, a mixture of 100 g of a purified TAL, BENT, PT or RB powder, 1.5 g calcium carbonate (adjusted to neutral pH) and 1 g carboxy methyl cellulose (CMC adhesive) was added under sterile conditions, following the method described by Vidhyasekaran and Muthuamilan (1995). The product was shade dried to reduce the moisture content (less than 20%) and then packed in polypropylene bags and sealed.

Evaluation of bioformulations on sugar beet seedlings growth characteristics

For seed treatment, sugar beet seeds were initially surface-sterilized with 1% sodium hypochlorite, washed with sterile

Table 1. Biodeveloped and used in the study and their ingredients

Bioformulation	Ingredients
Bent.-B1	Suspension of *P. fluorescens* (40 ml) containing 10^9 CFU ml^{-1} mixed with fine grade bentonite(100 g) and CMC (1g).
Bent.-B2	Suspension of *B.coagulans* (40 ml) containing 10^0 CFU ml^{-1} mixed with fine grade bentonite (100 g) and CMC (1g).
Talc-B1	Suspension of *P. fluorescens* (40 ml) containing 10^9 CFU ml^{-1} mixed with fine grade talc (100 g) and CMC (1g).
Talc-B2	Suspension of *B. coagulans (*40 ml) containing 10^9 CFU ml^{-1} mixed with fine grade talc (100 g) and CMC (1g).
Peat-B1	Suspension of *P. fluorescens* (40 ml) containing 10^9 CFU ml^{-1} mixed with peat powder (100 g) and CMC (1g).
Peat-B2	Suspension of *B.coagulans* (40 ml) containing 10^9 CFU ml^{-1} mixed with peat powder (100 g) and CMC (1g).
R.B.-B1	Suspension of *P. fluorescens* (40 ml) containing 10^9 CFU ml^{-1} mixed with rice bran powder (100 g) and CMC (1g).
R.B.-B2	Suspension of *B.coagulans* (40 ml) containing 10^9 CFU ml^{-1} mixed with rice bran powder (100 g) and CMC (1g).

CMC - carboxy methyl cellulose

Table 2. A comparison among different bioformulations in promoting sugar beet seedlings root length (SRL) (cm), root weight (RW) (g), height (SH) (cm), wet weight (SWW) (g) and dry weight (SDW) (g) 60 days after sowing in the greenhouse

Treatment	SRL (cm)	SRW (g)	SH (cm)	SWW (g)	SDW (g)
Control	11.50 c	0.33 d	30.75 d	5.36 d	0.39 d
Peat –b l	13.00 bc	1.38 b	49.00 ab	25.95 b	1.87 b
Peat –b2	16.13 a	2.10 a	56.25 a	24.72 b	2.10 ab
R.B.-b1	13.50 b	0.91 c	43.50 bc	19.31 c	1.46 c
R.B.-b2	13.90 b	1.92 a	47.00 ab	25.26 b	1.90 b
Bent.-b1	12.50 bc	2.17 a	49.00 ab	37.19 a	2.83 a
Bent.-b2	13.00 bc	1.15 bc	41.50 bc	18.17 c	1.48 c
Talc-b1	14.75 ab	1.59 b	51.50 ab	35.10 a	2.30 ab
Talc-b2	14.85 ab	1.10 bc	46.00 ab	19.64 c	1.57 bc

Each figure (value) is an average of four replicates.

In each column, values marked with the same letters are not statistically different according to Duncan Multiple Range test.

water and then were transferred to 20-cm diameter petri dishes containing one of prepared BF. Eighty seeds (twenty for each pot) were then rolled in the powder for about 5 minutes until their surface was completely coated with the BF. The above procedure was performed separately for each prepared bioformulation (Vidhyasekaran *et al.*, 1997).

Efficacy of BFs were then assessed 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 sugar beet field fields in Tehran province of Iran was air-dried, homogenized using a revolving jar mill and pasteurized using a steam heater for 3 h at 85°C. Pots (20 cm diameter) were filled with soil (3.5 kg). Twenty treated sugar seeds with bioformulations were sown (depth 2 cm; spacing 2×3 cm) in each pot. Control (soil + untreated seeds) was also included. Sixty days after sowing, growth characteristics of sugar beet seedlings were determined in different treatments by gently removing them from the soil and transferring them to the laboratory. In the laboratory, different growth factors including seedling height, seedling fresh weight; seedling dry weight, root length and root weight were measured following common procedures (Tennant, 1975; Molla *et al.*, 2001).

Statistical Analyses

Experiment was performed in 10 treatments each with 4 replications. Analyses of variance and comparison of means were done separately by Duncan multiple range tests using Co-Stat statistical software (Cohort Software Company, CA).

RESULTS

Table 1 indicates different BFs used in the study. In this table, all ingredients of BFs including the bacterial suspension, inorganic and organic carriers are shown and the details of their development and preparation are described.

The results of evaluation of BFs effects on seedlings growth parameters are shown in Table 2. As the table indicates, 5 out of 8 BFs with different extend increased seedlings root length significantly (compared to untreated control). Among them, Peat-B2 was the most effective followed by Talc-B2, Talc-B1, R.B.-B2 and R.B.-B1 ($P<0.05$). However, the remaining 3 BFs were not effective in promoting seedlings root length (Table 2). In regard with seedling root weight, as the table shows results are somewhat different. According to data presented in Table 2, all BFs showed significant effects in increasing seedlings root weight when compared to the control ($P<0.05$). In this section, Bent.-B1, Peat-B2 and R.B.-B1 showed the most effectiveness followed by Talc-B1, Peat-B1, Bent.-B2, Talc-B2 and R.B.-B1 (Table 2).

In addition to the above growth factors, seedlings height were also significantly promoted by all BFs with various degree of efficacy ($P<0.05$). Among them, Peat-b2 was the most effective followed by Talc-B1, Peat-B1, Bent.-B1, R.B.-B2, Talc-B2 and R.B.-B1 and Bent.-B2 (Table 2). Results of the effectiveness of BFs on seedlings wet weight indicated that all BFs affected seedlings fresh weight and increased it significantly ($P<0.05$) when compared to the control category. In this criterion, Bent.-B1 and Talc-B1 showed the highest effectiveness followed by Peat-B2, R.B.-B2, Peat-B1, and Talc-B1, R.B.-B1, Bent.-B2 respectively (Table 2). In addition to seedlings height and seedlings wet weight, seedlings fresh weight was also increased ($P<0.05$). As indicated in Table 2, fresh weight was promoted by prepared BFs with different ratio. In this section of the results, Bent.-B1, Talc-B1 and Peat-B2 were the most effective followed by R.B.-B2, Peat-B1, Talc-B2, Bent.-B1 and R.B.-B1 respectively ($P<0.05$).

DISCUSSION

The overall results of this study show that it may be possible to promote and increase sugar beet seedlings growth characteristics by development and application of some BFs. This is probably because these BFs play effective roles in increasing, establishment and durability of antagonistic microorganisms in the soil and possibly enhance production of antibiotics, siderophores, hydrolytic enzymes, phytohormones and/or other volatile extra-cellular metabolites (Chen *et al.*, 2000; Shahraki *et al.*, 2009).

In a previous study, Molla *et al*. (2001) evaluated the potential enhancement of root growth and nodulation in soybean with application of *Azospirillum brasilense* and *A. lipoferum* co-inoculated with two *Bradyrhizobium japonicum* strains. They observed significant root growth stimulation and nodulation in *Azospirillum* as well as during its co-inoculation with *Bradyrhizobium*. Total root length, root number, specific root length, root fresh matter, root hair development and shoot fresh matter were also significantly increased by *Azospirillum* alone and its co-inoculum. Similarly, Bharathi *et al.* (2004) evaluated the efficacy of 13 plant growth promoting rhizobacterial strains against chilli fruit rot and dieback incited by *Colletotrichum capsici*. Similar to our study, they also observed that among their test formulations, *P. fluorescens* and *B. subtilis* were more effective in increasing the seed germination and seedling vigor. They also found that the PGPR mixed bioformulation (*P. fluorescens* + *B. subtilis* + neem + chitin) was the most effective in reducing the fruit rot incidence, apart from increasing plant growth and yield parameters under both greenhouse and field conditions.

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. In our study although all BFs performed effectively in promoting sugar beet seedlings growth characteristics, but *P. fluorescens* based formulations were relatively more effective. This could probably be due to the more diverse metabolites produced by this bacterium (siderophore, hydrolytic enzymes, phytohormones and/or other volatile extra-cellular metabolites).

Among the carriers, peat, rice bran, talc and bentonite performed well and effective in their respective developed BFs. Furthermore, 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 agricultural system.

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