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Production of *Pseudomonas fluorescens* P-5 and P-6 for Bean Damping-off Disease

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ABSTRACT

Growth and efficacy of two biological control agents, *Pseudomonas fluorescens* Flügge, P-5 and P-6, were evaluated in combinations of two carbon (sucrose & molasses) and two nitrogen (urea & yeast extract) sources to optimize control of *Rhizoctonia solani* Kühn, which is a causal agent of bean damping-off. Both strains were grown in five liquid media including: sucrose + yeast extract, molasses + yeast extract (2:1 w/w), molasses + yeast extract (1:1 w/w), molasses + urea and nutrient broth at an initial inoculation of 1×10^5 CFU mL^{-1}. Cells from overnight cultures were used to inoculate soil at 1×10^9 CFU cm^{-3} soil. At the same time, fungal inoculum was added to soil at the rate of 2 mg cm^{-3} soil. The medium containing molasses and yeast extract (1:1 w/w) supported rapid growth and high cell yields in both strains. In greenhouse conditions, the influence of the media on the biocontrol efficacy of P-5 and P-6 was the same and *P. fluorescens* P-6 in molasses + yeast extract (in two different ratios) reduced the severity of disease from 90.9% to less than 28%. On the other hand, there were significant differences on bean growth promotion in greenhouse conditions, after one month. Strain P-5 in molasses + yeast extract (1:1 w/w) was more effective on bean growth promotion as compared to the other media.

Key Words: Production; *Pseudomonas fluorescens*; *Rhizoctonia solani*; Growth; Biocontrol efficacy

INTRODUCTION

Alternative strategies for disease management include the use of bacteria that show benefic effects on plants and these bacteria are known as plant growth-promoting rhizobacteria (PGPR). The positive effects of PGPR are normally divided into two categories: growth promotion and biological control (Kloepper, 1997). Also, certain root-colonizing bacteria can protect plants from soil-borne pathogens when used as inoculants (Keel *et al*., 1989; Slininger *et al*., 1996). However, the properties of the formulation used to deliver these bio-control agents can influence the success of the inoculation (Shah-Smith & Burns, 1997). Formulation of bio-control agents have been designed to promote their survival in soil (Trevors *et al*., 1992) and colonization of the rhizosphere, effective disease suppression (Slininger *et al*., 1996).

Most of the bio-control strains such as PGPR bacteria have varied performance in different environmental conditions. Some of this variability has been attributed to differences in physical and chemical properties of the natural environments, where bio-control agents are applied (Thomashow & Weller, 1996; Duffy *et al*., 1997). The laboratory medium has a profound effect on bio-control agents and products, including ability to grow and effectiveness in disease control. The accurate incorporation of nutrients can improve the biomass production of bio-control agents, but unexpectedly did not enhance (Slininger *et al*., 1996) or even decreased the bio-control efficacy (Moënne-Loccoz *et al*., 1999). On a large scale, the medium should allow a maximum concentration of biomass and a high quality culture to be produced at a low price (Lewis, 1991).

The aim of the present study was to find the carbon and nitrogen sources that should provide maximum biomass production of *P. fluorescens* P-5 and P-6 with minimum cost of media, whilst maintaining or promoting bio-control efficacy.

MATERIALS AND METHODS

Antagonists and pathogen preparation. From seven strains of *P. fluorescens*, only two strains P-5 and P-6 were selected for this investigation (Table I). These strains showed the inhibitory zone in a dual culture assay against *R. solani* on potato dextrose agar (PDA). Both of the strains were grown weekly on starch agar (Costa *et al*., 2001) plates containing (per liter): 5 g peptone, 5 g yeast extract and 3 g soluble starch. After growing for 24 h at 27˚C, the plates were stored at 4˚C. *R. solani* was grown at 24˚C on potato dextrose agar. Microorganisms belong to the laboratory of biological control of University of Tehran, Iran.

Inoculation and growth condition. Both of the strains were cultured in 250 mL conical flasks, using 50 mL of

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each autoclaved medium (in all liquid cultures, pH adjusted to 6.9). The flasks were inoculated with fresh cultures of each of the two strains at an initial concentration of 1×10^5 colony-forming units (CFU) mL^{-1} and were incubated at 27°C under agitation (140 rpm). Samples were taken after 20 h of incubation; populations of two stains were estimated in duplicate on starch agar, using the surface-plated method. The sample dilutions were made in sterile distilled water and the plates were incubated at 27°C for 24 h. The viable populations (CFU mL^{-1}) were recorded. Each flask assay was conducted in three replicates and two independent assays were carried out for each medium.

Culture media. In the experiments, five different medium containing: (1) sucrose (l0 g L^{-1}) + yeast extract (5 g L^{-1}), (2) molasses (20 g L^{-1}) + yeast extract (10 g L^{-1}), (3) molasses (20 g L^{-1}) + urea (2 g L^{-1}), (4) molasses (12 g L^{-1}) + yeast extract (12 g L^{-1}), (5) nutrient broth (NB) (8 g L^{-1}) were used.

Greenhouse conditions. Bacterial strains P-5 and P-6 were grown in 50 mL of five media and they were harvested at 20 h and centrifuged for two times at 6.981 g for 10 min. The supernatants were discarded and the bacterial pellet in each centrifuged tube was resuspended in 50 mL sterile distilled water and homogenized. The concentrations of suspension were used to inoculate soil at 1×10^9 colony forming units (CFU) cm^{-3} soil. At the same time, fungal inoculum (infected millet seed with *R. solani* was added to soil at the rate of 2 mg cm^{-3} soil. Data were recorded as the percentage of disease index (after two weeks) and the fresh weight of bean (after one month). Four replicates (containing three plants in each replicate) were used per treatment and the experiment was carried out twice.

Statistical analysis. Statistical analysis was performed by using MSTATC soft ware. Data for final populations (CFU mL^{-1}) and the incidence of damping-off was studied by an analysis of variance applied to $\sqrt{x+½}$, followed by Duncan's test for separation of means when the variable was statistically significant ($P\leq0.01$).

RESULTS

Control of *R. solani* with seven bacterial antagonists in dual culture assay. The results of this experiment are presented in Table II. From seven bacterial antagonists tested, *P. fluorescens* P-5 and P-6 were more effective in controlling *R. solani* than the other strains.

Growth assay. The medium containing molasses and yeast extract (1:1 w/w) supported rapid growth and high cell yields in both of the strains. Growth of P-6 in this medium was significantly higher (3.7×10^9 CFU mL^{-1}) than the other media. The final growth of P-5 in two media containing molasses and yeast extract in two different ratio (1:1 & 2:1 w/w) was significantly higher than in the other media used (2.3×10^9 & 2.2×10^9 CFU mL^{-1}). The combination of molasses + yeast extract for both of the strains was more effective on their final growth as compared to the other media (Fig. 1).

Table I. Description of bacterial strains (*P. fluorescens*)

Strain	Plant*	Place[α]
P-5	Onion	Ghareh Tapeh, Iran
P-6	Pea	Karaj, Iran
P-8	Pistachio	Kerman, Iran
P-14	Wheat	Karaj, Iran
P-30	Wheat	Karaj, Iran
P-35	Wheat	Karaj, Iran
P-59	Onion	Ghareh Tapeh, Iran

*The plant that bacterial strain was isolated from it
[α]The place that bacterial strain was isolated.

Table II. Inhibition halos for seven strains of *P. fluorescens* P-5 and P-6 against *R. solani*. Bacterial strains were tested in potato dextrose agar (PDA)

Bacterial strain	Inhibition halo (mm)*	Duncan's[α]
P-5	16.2	a
P-6	14	b
P-8	0	f
P-14	6.3	e
P-30	6.4	e
P-35	9	c
P-59	7	d

* Means for bacterial antagonists are resulted from three replicates.
[α] Means followed by the same letter are significantly equal, according to Duncan's multiple range test ($P\leq0.01$).

Bio-control efficacy assay in greenhouse. All the strains significantly inhibited pathogenicity of *R. solani* in greenhouse conditions and molasses media showed good yield with both of the strains. In the greenhouse conditions, the influences of five media on the bio-control efficacy of P-5 and P-6 were the same. *P. fluorescens* P-6 in molasses + yeast extract (1:1 & 2:1 w/w) reduced the severity of disease from 90.9% to less than 28%. Also, *P. fluorescens* P-5 reduced the severity of disease to less than 46%, in all of media used (Fig. 2).

Measurement of fresh weight of bean. Significant differences on growth promotion of bean among the treatments after one month were observed. Strain P-5 in molasses + yeast extract (1:1 w/w) had the highest effect on fresh weight (shoot & root) promotion of bean, whereas this strain had the least effect on growth of bean in the medium containing molasses and urea. Generally the function of P-5 on growth promotion of bean in all of the media was the same (Fig. 3). Strain P-6 was more efficient in fresh weight (shoot & root) production of bean in molasses + yeast extract (2:1 w/w) and there was a significant difference between molasses + yeast extract (2:1 w/w) medium and NB (Fig. 4).

DISCUSSION

This study proved that the nature and relative concentration of carbon and nitrogen sources in culture media are important for the growth and production of bio-control agents. Molasses used in our experiment contained about 50% sucrose and the raw material used for sugar production was sugar beet. This high sucrose concentration may justify the high biomass obtained, because the

Fig. 1. Effect of five different media on growth of *P. fluorescens* P-5 and P-6. Growth was carried out in 50-mL conical flasks at 27°C, shaken at 140 rpm for 20 h. Five different media including: Sucrose (10 g L^{-1}) + yeast extract (5 g L^{-1}; Suc.yeast), molasses (20 g L^{-1}) + yeast extract (2:1 w/w) (10 g L^{-1}; Mol.yeast 2:1), molasses (20 g L^{-1}) + urea (2 g L^{-1}; Mol.urea), molasses (12 g L^{-1}) + yeast extract (1:1 w/w) (12 g L^{-1}; Mol.yeast 1:1), NB (8 g L^{-1}). The separations of means according to Duncan's test are shown for every medium ($P \leq 0.01$). Columns with different letters indicate differences between media. CFU Colony-forming units

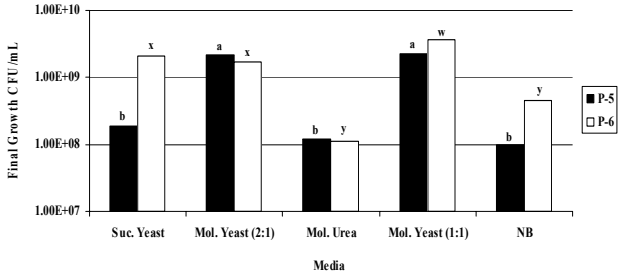
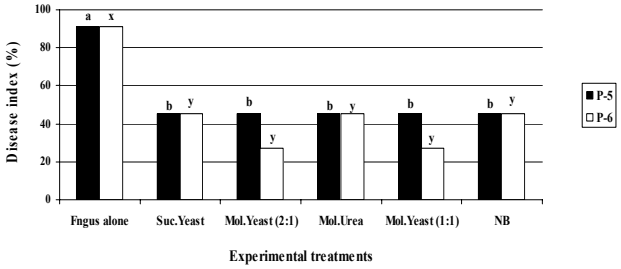


Fig. 2. Effect of five different media on biocontrol efficacy of P-5 and P-6 of *P. fluorescens* against *R. solani*. Cultures containing about 1×10^9 CFU ml^{-1} and growing in five different media: Sucrose (10 g L^{-1}) + Yeast extract (5 g L^{-1}; Suc.yeast), molasses (20 g L^{-1}) + yeast extract (2:1 w/w) (10 g L^{-1}; Mol.yeast 2:1), molasses (20 g L^{-1}) + urea (2 g L^{-1}; Mol.urea), molasses (12 g L^{-1}) + yeast extract (1:1 w/w) (12 g L^{-1}; Mol.yeast 1:1), NB (8 g L^{-1}). The statistical relationship between treatments is indicated with different letters, according to Duncan's multiple rang test ($P \leq 0.01$).



combination of yeast extract and commercial sucrose also showed a high final growth of bacteria. Also, the low cost of molasses allows its concentration to be increased in media without reducing its benefic effects on production and bio-control ability of bacterial antagonists for commercial use. However, higher concentration of this substrate (40 g L^{-1}) did not significantly improve bacterial growth, so a high concentration of this carbon source could be toxic to cells of the bio-control agents (Costa *et al*., 2001). Luna *et al*. (2002) and Costa *et al*. (2001) demonstrated that the molasses-based medium was considerably good for the purpose of bio-control.

Fig. 3. Effect of five different media on fresh weight promotion of bean. *P. fluorescens* P-5 growing in five different media: Sucrose (10 g L^{-1}) + yeast extract (5 g L^{-1}; M1), Molasses (20 g L^{-1}) + yeast extract (2:1 w/w) (10 g L^{-1}; M2), molasses (20 g L^{-1}) + urea (2 g L^{-1}; M3), molasses (12 g L^{-1}) + yeast extract (1:1 w/w) (12 g L^{-1}; M4), NB (8 g L^{-1}; M5). The statistical relationship between treatments is indicated with different letters, according to Duncan's test ($P \leq 0.01$)

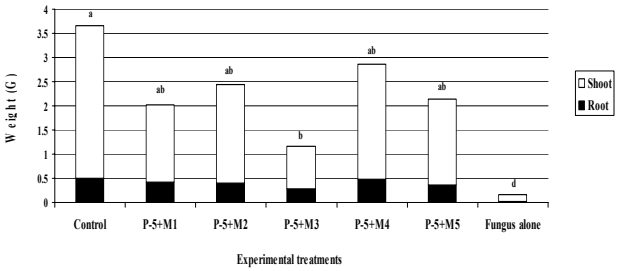
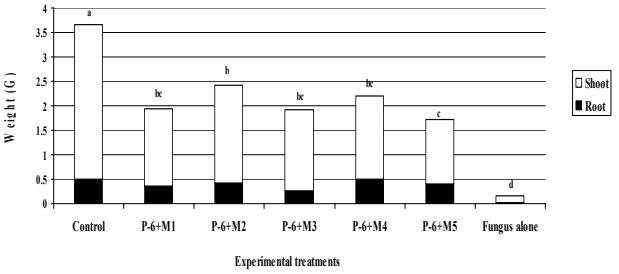


Fig. 4. Effect of five different media on fresh weight promotion of bean. *P. fluorescens* P-6 growing in five different media: Sucrose (10 g L^{-1}) + yeast extract (5 g L^{-1}; M1), molasses (20 g L^{-1}) + yeast extract (2:1 w/w) (10 g L^{-1}; M2), molasses (20 g L^{-1}) + urea (2 g L^{-1}; M3), molasses (12 g L^{-1}) + yeast extract (1:1 w/w) (12 g L^{-1}; M4), NB (8 g L^{-1}; M5). The statistical relationship between treatments is indicated with different letters, according to Duncan's test ($P \leq 0.01$)



The use of yeast extract as a nitrogen source supports rapid growth and higher cell yields in all of the strains as compared to urea. In fact, urea was not a good supplement for molasses in this study but yeast extract was a remarkable complement for it. The combination of molasses and yeast extract in two different ratio (1:1 & 2:1 w/w) provided considerable results in all of steps of this research. Molasses + yeast extract showed considerable growth and good yield with both of the strains, *in vitro* and *vivo* conditions. This combination for both of the strains was more effective on growth of bean in greenhouse conditions. Yeast extract contains amino acids and peptides, water-soluble vitamins and carbohydrates (Crueger & Crueger, 1993). Costa *et al*. (2001) and Dharani-Aiyer (2004) demonstrated that yeast extract was the best organic nitrogen source for antagonist bacteria. Nohata and Kurane (1997) considered yeast extract too expensive for an industrial process, thus it should be

replaced with another industrial product instead of yeast extract, which is confirmed by an economic and technological study.

In greenhouse conditions, the influence of laboratory medium on the bio-control efficacy of strains involved the interactions of the antagonist agents with the resident soil microbiota. Fuchs *et al*. (2000) hypothesized that the laboratory medium had an effect on the physiological state of the cells, which influenced the subsequent bio-control ability of strains in the rhizosphere.

This study demonstrated the importance of nutrition on growth promotion of bean as well. The medium containing molasses and yeast extract had a considerable effect on growth-promotion of bean. So, it could be inferred that the media used in this study not only had no negative impression in PGPR bacteria but also were more effective on growth promotion of bean in some treatments as compared to NB.

Although by-products such as molasses have a low price, this may have some disadvantages that must be taken into account. It is not as standardized as purified products and also contains impurities that are removed before fermentation (Stanbury *et al*., 1995). Moreover, their composition may vary according to the season and origin. This study has demonstrated that antagonist bacteria such as *P. fluorescens* can be produced in different media, using various nitrogen and carbon sources, whilst maintaining the efficacy of the bio-control agents. The results obtained in this study could be used to provide a reliable basis to increase of population of bio-control agents in fermentation process.

CONCLUSION

The laboratory conditions used to prepare the inoculum need to be considered carefully when optimizing production of a bio-control of a pseudomonad. Improvement in bio-control efficacy may be achieved when richer laboratory media are replaced with less rich ones, which in addition may lower manufacturing costs of bio-control products.

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REFERENCES

Costa, E., N. Teixdó, J. Usall, E. Atarés and I. Viñas, 2001. Production of the biocontrol agent *Pantoea agglomerans* strain CPA-2 using commercial products and by-products. *Appl. Microbiol. Biotechnol.*, 56: 367–71

Crueger, W. and A. Crueger, 1993. *Substratos Para La Fermentation Industrial*, Acribia, Madrid

Dharani-Aiyer, P.V., 2004. Effect of C: N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27. *African J. Biotechnol.,* 10: 519–22

Duffy, B.K., B.H. Ownley and D.M. Weller, 1997. Soil chemical and physical properties associated with suppression of take-all of wheat by *Trichoderma koningii*. *Phytopathol.,* 87: 1118–24

Fuchs, J.G., Y. Moënne-Loccoz and G. Défago, 2000. The laboratory medium used to grow bio-control *Pseudomonas* sp. Pf153 influences its subsequent ability to protect cucumber from black root rot. *Soil Biol. Biochem.,* 32: 421–4

Keel, C., C. Voisard, C.H. Berling and G. Kahr, 1989. Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHAO under gnotobioticconditions. *Phytopathol.*, 79: 584–9

Kloepper, J.W., 1997. Current status and future trends in bio-control research and development in the U.S. *In: 1997 Int. Symp. on Clean Agriculture, Sapporo, OECD*, pp: 49–52

Lewis, J.A., 1991. Formulation and delivery systems of bio-control agents with emphasis on fungi. *In:* Keister, D.L. and P.B. Cregan (eds.), *The Rhizosphere and Plant Growth*, pp: 279–87. Rotterdam, Kluwer

Luna, C.L., R.L.R. Mariano and A.M. Souto-Maior, 2002. Production of a bio-control agent for crucifer's black rot disease. *Brazil J. Chem. Eng.,* 19: 133–40

Moënne-Loccoz, Y., M. Naughton, P. Higgins, J. Powell, B. O'Connor and F. O'Gara, 1999. Effect of inoculum preparation and formulation on survival and bio-control efficacy of *Pseudomonas fluorescens* F113. *J. Appl. Microbiol.,* 86: 108–16

Nohata, Y. and R. Kurane, 1997. Complete defined medium for large-scale production of polysaccharide biosorbent from *Alcaligenes lactus* B-16. *J. Ferment. Bioeng.,* 83: 116–7

Shah-Smith, D.A. and R.G. Burns, 1997. Shelf-life of a bio-control *Psuedomonaas putida* applied to sugar beet seeds using commercial coatings. *Bio-control Sci. Technol.*, 7: 65–74

Slininger, P.J., J.E. Van Cauwenberge, R.J. Bothast, D.M. Weller, L.S. Thomashow and R.J. Cook, 1996. Effect of growth culture physiological state, metabolites and formulation on the viability, phytotoxicity and efficacy of the take-all bio-control agent *Pseudomonas fluorescens* 2-79 stored encapsulated on wheat seeds. *Appl. Microbiol. Biotechnol.,* 45: 391–8

Stanbury, P.F., A. Whitaker and S.J. Hall, 1995. Media for industrial fermentations. *In:* Stanbury, P.F., A. Whitaker and S.J. Hall (eds.), *Principles of Fermentation Technology*, pp: 93-121. Oxford, Pergamon Press

Thomashow, L.S. and D.M. Weller, 1996. Current concept in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. *In:* Stacey, G. and N.T. Keen (eds.), *Plant-Microbe Interactions*, pp: 187–235. New York

Trevors, J.T., J.D. Van Elsas, H. Lee and L.S. Van Overbeek, 1992. Use of alginate and other carriers for encapsulation of microbial cells for use in soil. *Microbial. Release*, 1: 61–9

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