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Full Length Research Paper

Influence of some culture media on antifungal activity of *Pseudomonas fluorescens* UTPF61 against the *Sclerotinia sclerotiorum*

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Fluorescent pseudomonads are ubiquitous bacteria that are common inhabitants of the rhizosphere and are the most studied group within the genus *Pseudomonas*. Strain UTPF61 is a biocontrol agent against *Sclerotinia* wilt of sunflower, an important disease of sunflower caused by *Sclerotinia sclerotiorum*. This bacterium was selected out of 47 strains. It was grown in eight culture media containing different carbon sources, such as sucrose, glucose, corn steep liquor, fructose, glycerol, sugar beet molasses, manitol and starch. Our results showed that the starch has a considerable effect (*P<0.01*) on the rate of bacterial growth as compared to the other carbon sources used. Investigation of antifungal activity *in vitro* indicated that some media containing glucose and sugar beet molasses were the most effective (*P<0.01*) on antagonistic efficiency, whereas the medium containing sugar beet molasses was the most effective on antagonistic efficiency in greenhouse trials (with 87% healthy plants).

Key words: Antagonistic efficiency, antifungal activity, biological control, carbon sources, growth rate.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a plant pathogenic fungus that causes destructive diseases of numerous succulent plants (Agrios, 2005). Sunflower (*Helianthus annus* L.) is vulnerable to some pathogenic agents especially the fungus *S. sclerotiorum.* There has been no progress in breeding a type of sunflower resistant to *sclerotinia* wilt and no effective chemical control of this disease is known (Expert and Digat, 1995). Interest in biological control has recently been intensified because of imminent bans on effective chemical controls such as methyl bromide, widespread development of fungicide resistance in pathogens, and a general need for more sustainable disease control strategies (Duffy and Defago, 1999). Biocontrol has become a great potential for an alternative method of disease control (Weller, 1988). Some fluorescent pseudomonads are referred to as plant growth-promoting rhizobacteria (PGPR) and their effectiveness in controlling a number of plant diseases caused by soil-borne pathogens has been widely documented (Sullivan and Gara, 1992; Glick, 1995). The inoculation of seeds or roots with fluorescent pseudomonads to increase plant vigour and productivity has been a worldwide studied practice (Kloepper et al., 1980). Some strains of *P. fluorescens* were reported as biocontrol agents of *S. sclerotiorum* (Expert and Digat, 1995). Unfortunately, most of the biocontrol strains have inconsistent performances under different conditions. Some of this variability has been attributed to differences in physical and chemical properties that occur in natural environments where biocontrol agents were applied (Duffy et al., 1997; Thomashow and Weller, 1996).

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Table 1. Some bacteria isolates used in the study.

Strain		Host	Sources
UTPF	*P.fluorescens*	Canola	Unknown
UTPF10	*P. flourescens*	Wheat	Karaj
UTPF18	*P. flourescens*	Wheat	Karaj
UTPF24	*P. flourescens*	Wheat	Karaj
UTPF30	*P. flourescens*	Wheat	Karaj
UTPF32	*P. flourescens*	Wheat	Karaj
UTPF45	*P. flourescens*	Bean	Khomein
UTPF54	*P. flourescens*	Bean	Khomein
UTPF59	*P. flourescens*	Onion	Gharetape
UTPF60	*P. flourescens*	Rice	Shamokalmot
UTPF61	*P. flourescens*	Rice	Almot of Ghazvin
UTPF63	*P. flourescens*	Canola	Mazandaran
UTPF65	*P. flourescens*	Canola	Mazandaran
UTPF75	*P. flourescens*	Rice	Almot
UTPF76	*P. flourescens*	Rice	Almot
UTPF81	*P. flourescens*	Rice	Ghasinrod
UTPF82	*P. flourescens*	Rice	Almot
UTPF83	*P. flourescens*	Rice	Almot
UTPF85	*P. flourescens*	Rice	Gazarkhan
UTPF86	*P. flourescens*	Rice	Shamok
UTPF87	*P. flourescens*	Rice	Razmiyan
UTPF90	*P. flourescens*	Rice	Shamok
UTPF92	*P. flourescens*	Rice	Almot
UTPF93	*P. flourescens*	Rice	Almot
UTPF94	*P. flourescens*	Unknown	India
UTPF95	*P. flourescens*	Unknown	India

'Understanding which environmental factors are important and how these influence disease suppression is widely recognized as a key to improvement the level and reliability of biocontrol' (Duffy and Defago, 1999). Macro and micro elements both in natural environments and culture medium are one of the most important environmental factors. Adaptation to proper culturing medium can alter biocontrol ability and physiological state, and we must consider laboratory media in optimizing the use of biocontrol agents (Bae et al., 2007). In addition, one impediment to commercialization is the lack of liquid culture technology for inexpensive mass production and storable biocontrol agents (Slininger and Jackson, 1992). 'Use of commercial products or by-products from food industries, such as nitrogen sources, has tended to meet most of the above criteria for production media because they are cheap substrates' (Costa et al., 2001). However, the presence of undesirable products or problems of non-homogeneity in the by-products can limit their use in an industrial process (Costa et al., 2001). The aim of this study was to find the carbon sources that provide maximum biomass production of *P. fluorescens UTPF61*, improving the biocontrol potentiality against the *S. sclerotiorum*, optimizing the mass production process, and maintaining biocontrol efficiency on a large scale.

MATERIALS AND METHODS

Source of microorganisms and cultural conditions

This study was conducted in The Laboratory of Biological Control of Plant Disease, College of Agriculture of Karaj, University of Tehran, Iran. 26 strains of fluorescent pseudomonads used (Table 1) were kindly provided by the Laboratory of Biological Control and 21 strains were isolated from rhizospher of sunflower in three fields situated in Ghom. The bacteria were stored in 0.1 M magnesium sulfate hydrate ($MgSO_4.7H_2O$) solution at room temperature. The isolates were cultivated in nutrient broth (Merck, Germany) and stored in broth containing 15% glycerol at -20°C for short-term preservation. For the preparation of bacteria, a starter culture was grown on nutrient broth in tubes and incubated 48 h at 25°C in darkness.

Plate assay

Antifungal activity

All the above-mentioned bacteria were grown overnight in TSB broth, and 5 µl of each culture was spotted in plates containing

Table 2. Production of antimicrobial metabolite: Production of HCN (another strains, that were not mentioned, did not produce HCN), production of protease (production of clearing zone, another strains, that were not mentioned, did not produce protease) and *in vitro* activity of the best strains against the *S. sclerotiorum*.

Strain	HCN [a]	Strain	Protease [b]	Strain	Inhibition zone(mm) [c]	
UTPF61	+	UTPF61	+	UTPF61	21	a
UTPF32	+	UTPF92	+	UTPF93	20.3	ab
UTPF2	+	UTPF45	+	UTPF92	19.5	abc
UTPF75	+	UTPF10	+	UTPF60	17.67	abc
UTPF87	+	UTPF88	+	UTPF59	17	bc
UTPF95	+	UTPF59	+	UTPF83	16	c
UTPF93	+	UTPF93	+	UTPF82	16	c
UTPF18	+	UTPF90	+	UTPF81	12	d
UTPF83	+	UTPF65	+	UTPF76	11	de
UTPF82	+	UTPF75	+	UTPF90	7.97	ef
UTPF85	+	UTPF24	+	UTPF45	6.03	fg
UTPF88	+	UTPF95	+	UTPF94	5.03	fgh
UTPF86	+	UTPF76	+	UTPF30	3.97	hig
UTPF81	+	UTPF87	+	UTPF86	3.03	hig
UTPF65	+	UTPF30	+	UTPF95	3	hig
UTPF63	+	UTPF63	+	UTPF63	2	hi
UTPF10	+	UTPF82	+	UTPF75	1.03	i
UTPF60	+	UTPF83	+	UTPF88	1	i
UTPF90	+	UTPF60	+	UTPF87	1	i
UTPF59	+	UTPF81	+	UTPF54	1	i
UTPF76	+	UTPF32	+			
		UTPF54	+			
		UTPF18	+			
		UTPF86	+			

[a] Cyanid caused the indicator paper to turn blue; [b] protease produced cleaning zone in SAM media; [c] width of the zone of inhibition was measured from the edge of the fungal colony to the edge of the bacterial colony; *statistical significance was judged at the *P<0.01* level.

potato dextrose agar (PDA), 0.5 cm off the edge of the plates (four spots per plate). One hour later, seven-millimeter discs of fungus mycelia from 5 day grown cultures on PDA were placed in the center of the plates. Plates were incubated at 20°C for five days and examined for the evidence if the growth of the fungus was inhibited by the bacteria. A positive response was any visible zone of inhibition around the fungus, regardless of the size of the zone (Hagedorn et al., 1989). All the 47 bacterial isolates were tested in three independent replicates.

Production of antifungal metabolites

Each isolate was tested directly for the production of hydrogen cyanide and protease. Protease activity (casein degradation) was determined from cleared zones observed in skimmed milk agar (SMA) according to Nielsen et al. (1998). Production of Hydrogen cyanide (HCN) was detected by growing bacteria for 16 h at 24°C on plates containing NA with an indicator paper inoculated with 5 mg of copper (II) ethylacetoacetate, 5 mg of methylene bis(-n-n-dimethylaniline), and 2 ml chloroform, as described by Castric and Castric (1983). Production of cyanide caused the indicator to turn blue (Table 2).

Plant assay

Plant growth promotion in greenhouse trials

25 Isolates that had the greatest antagonistic effect against *S. sclerotiorum* in the plate assay were studied further in greenhouse trials. They showed different capabilities in the production of antimicrobial metabolites and promotion of the plant growth. Surface-sterilized (2.5% NaOCl, 5 min) sunflower seeds, Azargol, were pregerminated in moist chambers at 27°C for 48 h. Sterility of seeds was proved by a print on nutrient agar. Pregerminated seeds were bacterized by soaking in bacterial suspension at 10^8 cfu/ml (nutrient broth, grown for 16 h) for 30 min with a mild shaking. Bacterial suspension was prepared by 1% carboxyl methyl cellulose (CMC) solution. Control seeds were shaken in sterile 1% CMC solution for 30 min. Then bacterized seeds were planted in sterile perlite and irrigated by nutrient solution. Pots were kept in a greenhouse under the following conditions: 22± 3°C, 60% relative humidity and a photoperiod of 14 h light: 10 h darkness. The length of root and stem, the wet and dry weight of root and stem were calculated after 30 days after sowing. Each isolate was tested in 4 replicates. After these tests, strain UTPF61 was selected for the next experiments.

To determine growth curve of P. fluorescens UTPF61 in two media

Growth of *P. fluorescens* UTPF61 cells was tested in 250 ml Erlenmeyer flasks containing 50 ml of each media. Media used in this study were nutrient broth and king's B culture. To initiate this test, media were autoclaved for 15 min at 121°C. Then initial concentrations of 3×10^6colony-forming units (cfu) ml^{-1} were placed onto each sterile media and were incubated at 25°C under agitation (120 rpm). Samples were collected hourly for 48 hours, starting from inoculation, for measuring OD at A_{600}.Three replicate flasks were used. Regression analysis was performed using SIGMA PLOT software (version 11), and a growth curve was generated relating OD at A_{600}values (OD_{600}) to the time after inoculation. The aim of this test was to indicate the time the bacteria rate growth peaks, as to be used later to know when to collect the samples in next tests.

Effect of culture media on biocontrol activity of P. fluorescens UTPF61

The basic medium used in this study was a semi-defined medium (M1), containing in grams per liter: yeast extract, 1; sucrose, 10; $CaCO_3$, 0.4; $MgSO_4$, 0.4; $K_2HPO_4.3H_2O$, 0.98. In order to investigate the effect of different additional carbon sources, different carbon sources (tested at $4gl^{-1}$) including sucrose, glucose, corn steep liquor, fructose, glycerol, sugar beet molasses, manitol and starch were added to M_1 medium separately. In all cases, the medium were autoclaved at 121°C for 15 min. Bacterial growth was carried out in 100 ml conical flasks, using 50 ml of each autoclaved medium (described formerly). The flasks were inoculated with fresh cultures of strain UTPF61 at an initial concentration of 1×10^6 CFU ml^{-1} and were incubated at 25°C under agitation (120 rpm). Samples were taken after 48 h of incubation. Afterwards cell density was measured at 600 nm and cultures were used for dual culture assays and greenhouse studies.

***In vitro* antagonistic activity**

The *in vitro* inhibition of mycelial growth of *S. sclerotiorum* by the strain UTPF61 was tested using the dual culture technique as described by Keel et al. (1996). Three 10 µl drops from different flask cultures were equidistantly placed on the margins of PDA plates and incubated at 20°C for 24 h. Afterwards a 7 mm agar disc from fresh PDA cultures of *S. sclerotiorum* was placed at the centre of the PDA plate and incubated at 20°C for five days. Inhibition of fungal growth was assessed 5 days later by measuring the size of the inhibition zone (in mm).

***In situ* antagonism and plant growth promoting (PGPR) tests**

Experiments were performed in plastic pots (diameter, 7.5 cms; depth, 8) containing sterilized soil (sand 3, clay 1, humus 1). Surface-sterilized (5% sodium hypochlorite for 3 min and rinsed five times with sterile distilled water) and post germinated seeds of sunflower bacterized by soaking in bacterial suspension (1×10^8 CFU ml^{-1}, determined with hemasitometer lamella) for 30 min with a mild shaking. Bacterial suspension prepared by 1% CMC solution. Control seeds were shaken in sterile 1% CMC solution for 30 min. To prepare inocula of *S. sclerotiorum* for an antagonism test, millet seeds were sterilized (121°C, 20 min, twice), inoculated with mycelial plugs, and incubated at 20°C for 20 days. One gram of 24 h inoculated millet seeds was mixed into the upper part of sterile soil, prior to seed planting. For the control treatments, sterile millet seeds were mixed into the soil as mentioned before. In PGPR test, bacterized seeds were planted in sterile perlite and irrigated by nutrient solution. Pots were kept in a greenhouse under the following conditions: 22± 3°C, 60% relative humidity and a photoperiod of 14 h light: 10 h darkness. Pots were fully randomized in different places and treatments were replicated three times. In the antagonistic test, 14 days after planting, the percentage of healthy plants was determined according to the procedure described by Expert and Digat (1995) and in PGPR test, the length of root and stem, the wet and dry weight of root and stem was calculated after 20 days of sowing.

Statistical analysis

In order to compare trials, at least three replicates were used in each test as a check. No differences between corresponding experiments were found, so the trials were pooled and statistical analysis was performed for the pooled data using SAS software. Statistical significance was judged at the *P<0.01* level. When the analysis was statistically significant, Duncan's multiple tests range for separation of means was performed.

RESULTS

To select bacterial strain

P. fluorescens UTPF61 was selected out of 47 strains of fluorescent pseudomonads on the basis of dual culture assays, growth promotion activity on sunflower and production of HCN and protease (Heidary, 2008). Strain UTPF61 produced HCN and protease had the best effect on the antagonistic efficiency among the other bacteria (Table 2). Also among all the strains, this one had the best effect on PGPR (Table 3). This strain was already isolated from the rhizosphere of rice from Almot of Ghazvin, Iran.

To determine growth curve *of P. fluorescens* UTPF61 in two media

Regression analysis revealed a logarithmic relationship between the OD_{600} and the incubation time. Growth curve of bacteria in nutrient broth and King's B culture were almost similar to each other and maximum of growth happened nearly after 42 h (Figures 1 and 2). Therefore the following tests samplings were done after 48 h, because in this time bacteria had the maximum growth and produced some secondary metabolite.

Effect of culture media on biocontrol activity of *P. fluorescens* UTPF61

Bacterial growth rate

The estimation of the bacterial growth rate in different media showed that the medium containing starch was more effective on the rate of bacterial growth (Table 4).

Table 3. Effect of different strains of *P. flourescens* on growth of sunflower.

Strain	Length of root (cm)		Length of stem (cm)		Wet weight (g)		Dry weight (g)	
UTPF61	25	a*	31**	ab	4.03	cde	0.28	a
UTPF24	22.67	ab	28	abcde	4.43	abc	0.23	cde
UTPF90	21.33	bc	28.67	abcde	4.2	bcd	0.28	a
UTPF95	20	bc	28.3	abcde	3.47	efg	0.28	a
UTPF45	18	cd	29.3	abcd	4.03	cde	0.26	abc
UTPF2	18	cd	30.3	abc	4.06	cde	0.21	edf
UTPF63	18	cd	28.3	abcde	4.43	abc	0.23	cde
UTPF87	16.3	de	27	cdefg	2.8	h	0.17	ij
UTPF86	16	def	31.3	a	4.8	a	0.28	a
UTPF85	16	def	26.67	cdefg	3.7	def	0.22	cde
UTPF59	15.67	def	26.3	defgh	3.93	cdef	0.22	cde
UTPF93	15.67	def	23.3	ghi	2.63	h	0.19	efghi
UTPF18	14.67	defg	26	defgh	3.6	def	0.22	cde
UTPF60	14.67	defg	28.3	abcde	3.5	efg	0.2	efghi
UTPF83	14	efg	26	defgh	3.57	fe	0.21	defg
UTPF94	13.67	efg	30.3	abc	4.67	ab	0.26	ab
UTPF75	13.67	efg	26.3	defgh	3.47	efg	0.18	fghij
UTPF32	13.33	efg	25.67	defghi	3.9	cdef	0.23	cde
UTPF30	12.67	efg	31.3	a	4.8	a	0.24	bcd
UTPF76	12.67	efg	27.3	bcdef	3.7	def	0.21	defgh
UTPF81	12.67	efg	28	abcde	3.37	fg	0.18	ghij
UTPF88	12.33	fg	22.67	hi	2.67	h	0.18	ghij
UTPF92	11.67	g	25.3	efghi	2.7	h	0.17	hij
UTPF65	11	g	23.67	fghi	2.7	h	0.17	hij
UTPF54	11	g	25.67	defghi	2.97	gh	0.2	defghi
control	11	g	22	i	2.43	h	0.15	j

*Statistical significance was judged at the $P<0.01$ level; **the numbers are average of four replicates.

In vitro antagonistic test

S. sclerotiorum was more sensitive to strain UTPF61 when grown in the culture media containing glucose or sugar beet molasses compared with the culture media that is supplemented with corn steep or glycerol (Table 4).

In situ antagonism and plant growth promoting (PGPR) tests

Medium containing sugar beet molasses with 93.3% healthy plants showed more antifungal activity than the other culture media (Figure 3). In greenhouse plant growth promoting trials glucose was more effective in increasing the length of stem than the other media; starch was more effective in stimulating root length, and corn steep liquor was more effective in increasing the wet and dry weight of plant. They were significantly different with control treatment by 163.35, 181.81, 257.43 and 207.69%, respectively (Table 5).

DISCUSSION

Among PGPR, fluorescent pseudomonads occur commonly in the rhizosphere of plants and they are an important functional group of beneficial bacteria to control soilborne plant pathogens (Weller, 1988; Ellis et al., 2000; Fuchs et al., 2000; Ahmadzadeh and sharifi Tehrani, 2009). Since the commercial production of *P. fluorescens* to be applied as a biocontrol agent, requires both low cost and high cell density, the optimization of nutritional and environmental conditions to produce this bacterium are based on minimizing the cost and maximizing the cell density. The results obtained in this study showed that the nature of carbon source in culture media influenced the rate of bacterial growth but it is worth mentioning that the medium showing more effect on the rate of bacterial growth did not necessarily have the same effect on antagonistic efficiency, in other word, no correlation was found between rate of bacterial growth and antagonistic efficiency. This is in common with the findings by Duffy and Défago (2000) and Costa et al., (2001).

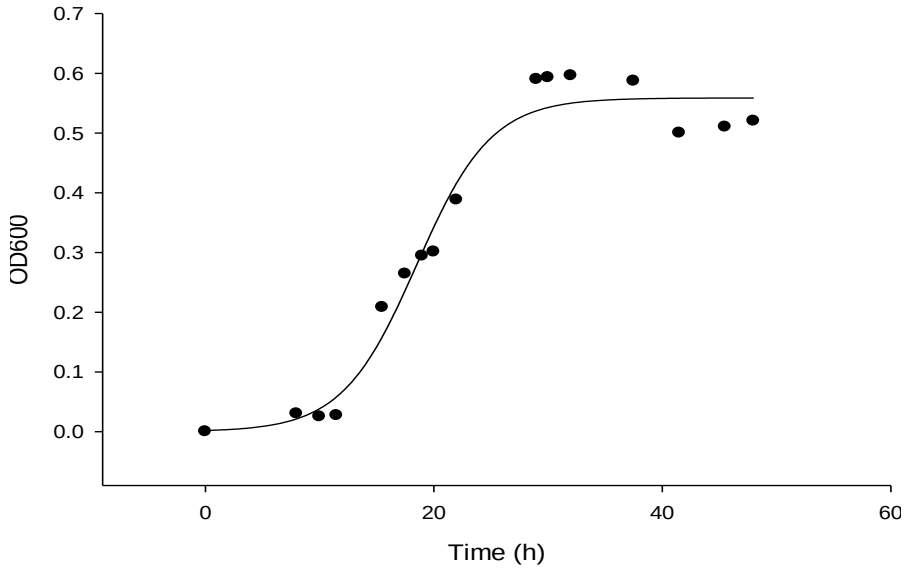

Figure 1. Growth curve *of P. fluorescens* UTPF61 in king's B culture. Samples were taken at 48 h for OD600 tests. This growth curve was from a representative of two repeat tests. The regression model is Y= a/(1+exp(-(x-x0)/b)) (r^2=0.9711, *P<0.0001*).

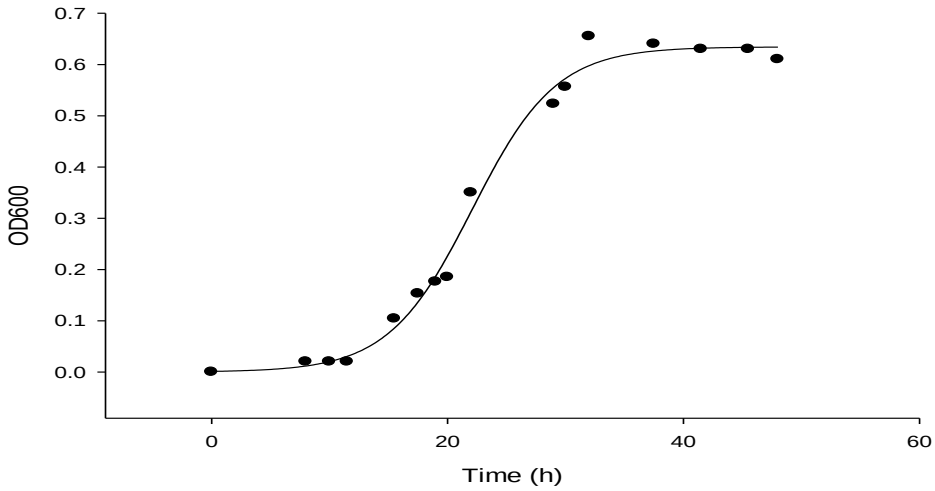

Figure 2. Growth curve *of P. fluorescens* UTPF61 in Nutrient broth culture. Samples were taken at 48 h for OD600 tests. This growth curve was from a representative of two repeat tests. The regression model is Y= a/(1+exp(-(x-x0)/b)) (r^2=0.9913, *P<0.0001*).

Biocontrol activity of bacteria is a result of secondary metabolite production. Approximately production of most of these secondary metabolites such antibiotics and siderophore increases in competition conditions such as in poor media. Siddiqui et al. (2002) and Duffy and Défago (1997) showed that addition of some of element such as zinc can increase biocontrol activity even in rich media. Conditions employed in the culture of biocontrol agents may have a significant influence on the production of antimicrobial substances (Duffy and Défago, 1999), survival in soil, and biocontrol ability (Fuchs et al., 2000; Gu and Mazzola, 2001). We also demonstrate here that cultural media can influence the biocontrol ability of *P. fluorescens* UTPF61. Medium containing glucose supports moderate cell yield of *P. fluorescens* UTPF61 but not as much as starch. Nevertheless, medium containing glucose, produce high antagonistic efficiency. Sugar beet molasses provided a moderate effect on bacterial growth but this medium has a considerable effect on growth factors of plant and produce high antagonistic efficiency and antifungal activity. Manitol and fructose provided the least bacterial growth but these media have a moderate effect on antagonistic efficiency. Molasses has been widely used in the production of

Table 4. Effect of some carbon sources on the growth rate and antagonistic efficacy of *P. fluorescens* UTPF61 in 25˚C and after 48 h.

Media	Inhibition zone(mm)		Optical density	
M1+ sucrose	11.33	bc*	0.48**	b
M1+corn steep liquor	10.33	c	0.56	b
M1+ glucose	14.33	a	0.52	b
M1+ fructose	10.67	bc	0.13	d
M1+ glycerol	10.33	c	0.2	d
M1+ sugar beet molasses	15.33	a	0.35	c
M1+ manitol	10.67	bc	0.1	d
M1+ starch	12	b	0.7	a

*Statistical significance was judged at the *P*<0.01 level.**OD600.

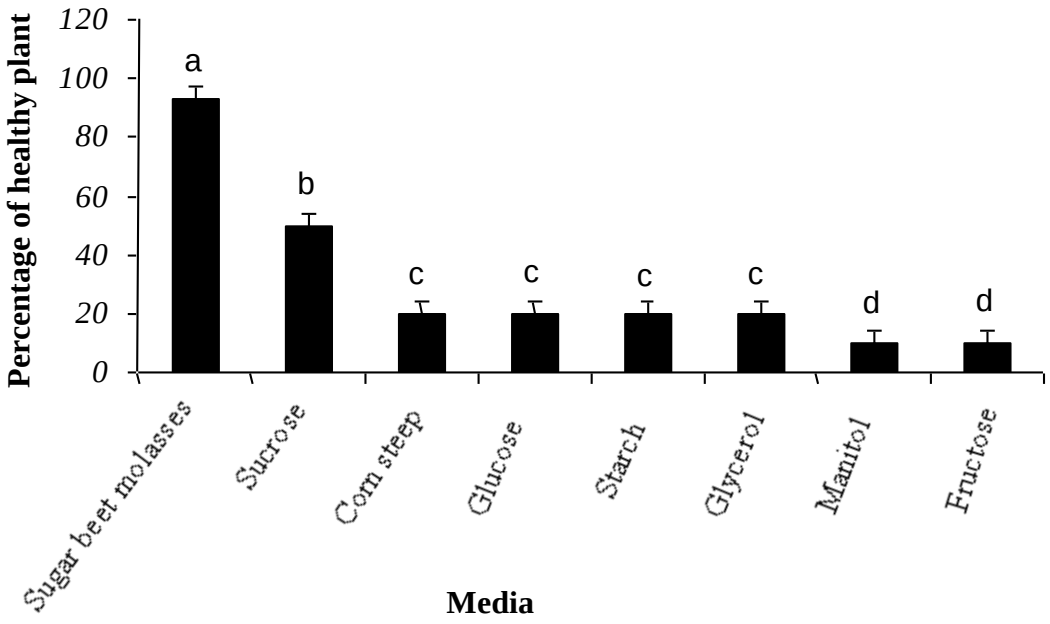

Figure 3. Percentage of healthy plant which treated with strain UTPF61 grown in different carbon sources.

microorganisms and was the best source that had the most effect on antagonistic efficiency.

This result is in agreement with Costa et al. (2001) and Luna et al. (2002). But this medium has some disadvantages that must be taken into consideration. By-products are not as standardized as purified products and they may contain impurities that need to be removed before fermentation (Costa et al., 2001). Moreover, their composition may vary according to the season and origin. Survival of *P. fluorescens* in soil can be influenced by the type of the laboratory medium used to grow the strain (Wessendorf and Lingens, 1989; Fuchs et al., 2000), and thus the biocontrol results obtained here might be explained by differences in survival of UTPF61 in soil and/or the rhizosphere. Colonization ability of in the host rhizosphere has long been considered as a key element to their successful use to control soilborne plant pathogens (Bae et al., 1990; Schippers et al., 1987; Weller, 1988). We hypothesized that cultural media can influence colonizational ability of biocontrol strains or/and indirectly affect the induction of systemic resistance in plants.

In conclusion, the laboratory conditions used to prepare the inoculums need to be considered carefully when optimizing production of a biocontrol pseudomonad. An improvement in biocontrol efficiency may be achieved when richer laboratory media are replaced with less rich media which, in addition, may lower manufacturing costs of biocontrol products. Ultimately it was an attempt to produce a media that is workable in mass production and financially rational; yet has the most efficiency in laboratory conditions. However, since this research has

Table 5. Effect of different carbon sources on ability of bacterium UTPF61 to improve growth of sunflower.

Bacteria obtained from different media	Length of root		Length of stem		Wet weight		Dry weight	
M1+ sucrose	18.67	ab	19.33	b*	2.73	b	0.21	b
M1+corn steep liquor	17	b	19.33	b	3.81	a	0.27	a
M1+ glucose	16.67	bc	22.33	a	2.85	b	0.23	ab
M1+ fructose	14.67	cd	19	b	3	b	0.21	b
M1+ glycerol	14.83	cd	19.67	b	3.11	b	0.22	ab
M1+ sugar beet molasses	13.3	d	19.5	b	2.92	b	0.23	ab
M1+ manitol	14.5	d	18.83	b	2.93	b	0.21	b
M1+ starch	20	a	19.33	b	2.76	b	0.19	b
Control	11	e	13.67	c	1.48	c	0.13	c

*Statistical significance was judged at the $P<0.01$ level.

not been performed in open farm conditions, it can be the subject of later experiments to be conducted in such places in order to obtain the most efficient mass production technology of nutrition supplements and also to improve fermentation process.

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