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Biological Control of *Gaeumannomyces graminis* var. *tritici* on Wheat with Some Isolates of *Pseudomonas fluorescens*

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Abstract: *Pseudomonas fluorescens* CHAO, *P. fluorescens* bioIII (21P), *P. fluorescens* bioIII (22P), *P. fluorescens* bioV(5 kM) and *P. fluorescens* bio V (32J) were evaluated as potential biological agent for wheat take-all caused by *Gaeumannomyces graminis* var. *tritici in vitro* and *vivo*. Dual culture, volatile metabolite and cell free culture test showed that all isolates of *Pseudomonas* tested inhibited growth of the pathogen. Inhibition varied from 32 to 73% in dual culture, from 52 to 96% in volatile metabolite and from 77 to 98% in cell free culture test. The seed soaking treatment with *P. fluorescens* CHAO and *P. fluorescens* bioIII (21P) were the most effective in reducing disease index and also promoted root and shoot weight in glasshouse and field experiment. The weight of 100 grain from plants treated with pathogen + *P. fluorescens* bioIII (21P) or *P. fluorescens* CHAO were significantly greater than in controls inoculated with pathogen alone in micorplot test. These results indicate that *P. fluorescens* bioIII (21P) could be an important new biological control for take-all of wheat.

Key words: Wheat disease, *Gaeumannomyces graminis* var. *tritici*, *Pseudomonas fluorescens*, biocontrol, soil borne disease

INTRODUCTION

Take-all of wheat caused by *Gaeumannomyces graminis*(Sacc.) Von Arx and Oliver var. *tritici* J.Walker (*Ggt*) is the most important crown and root diseases of wheat worldwide. Yield losses, due to stunting and premature ripening resulting in shriveled grain. Substantial financial losses result from decrease in yield and quality. Average of yield decrease reported about 1 t ha^{-1}(Cook *et al*., 2002). Chemical control of root diseases has almost always depended on the use of soil fumigants such as methyl bromide and chloropicrin. Obviously the cost of soil fumigation is not affordable for agronomic crop such as wheat. Treatment by Triadimenol and related compounds delay infection of *G.g.*var. *tritici*. The absence of useful genes for resistance to take-all in either wheat or barely is evidence of little or selection pressure on cereal species by this diseases. Biological control of naturally existing antagonistic microorganisms is an alternative strategy, which in certain circumstance might be integrated with other strategies. Numerous studies have demonstrated the ability of rhizosohere inhabiting microorganism to suppress disease caused by soil borne plant pathogens (Weller, 1988; Etebarian *et al*., 2000, 2003; Salehpour *et al*., 2005; Mazzola *et al*., 1995; Ashrafizadeh *et al*., 2005). A rang of different microorganism have been investigated as potential biological control agent for take-all (Duffy and Weller, 1995; Wong, 1994).

Particular attention has been paid to fluorecens pseudomonads, which have been demonstrated to provide effective biocontrol of take-all in pot and field experiment (Chapon *et al*., 2002; Desouza *et al*., 2003; Duffy and Weller, 1995; Ryder *et al*., 1990; Weller *et al*., 2002). Production of antifungal metabolites, such as antibiotic, hydrogen cyanide and siderophore-mediated iron competition, are primary mechanism by which these bacteria suppress disease (Leong, 1986; Leong and Gutterson, 1988).

Control of take-all is difficult where farmers wish to grow wheat continuously. Then biological control could become an alternative method of diseases control in this situation.

The purpose of this research was to evaluate the potential of some isolates of *Pseudomonas fluorescens* isolated from wheat rhizosphere in Mazandaran and Markazi Province, Iran for biological control of *Gaeumannomyces graminis* var. *tritici* the agent of wheat take-all and comparing this isolates with useful fungicide.

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MATERIALS AND METHODS

Pathogen and *Pseudomonas* isolates: *Pseudomonas fluorescens* bioIII (21P), *P. fluorescens* bioIII (22P) inhibited teletospore germination of *Tilletia laevis* (Khodayegan, 2003), *P. fluorescens* bioV (5 kM) reduced growth of *Bipolaris sorokiniana* (Mohamadi, 2004) and *P. fluorescens* bioV (32J) inhibited growth of *Fusarium graminearum* (Norouzian, 2003). All these bacteria isolated from rhizosphere of wheat and obtained from Department of Plant Protection, Abourayhan Campus, Tehran University. *Pseudomonas fluorescens* CHAO as a potential biological control of many root pathogens (Skaukat and Siddiqui, 2003) also used in this study for comparison. Isolate of *Gaeumannomyces graminis* var. *tritici* Ggt1 obtained by M.Ghalandar, Agricultural Research Center of Markazi province was used in this investigation. Bacterial and fungal isolates were maintained in sterilized distilled water (SDW) and potato dextrose agar (PDA) at 5°C, respectively.

Effect of *Pseudomonas* isolates on mycelial growth of *Ggt*: Dual culture (Dennis and Webster, 1971; Etebarian *et al*., 2003), cell free culture (Weller *et al*., 1988) and volatile metabolite tests (Fiddaman and Rossall, 1993) were used to observe the effect of *Pseudomonas fluorescens* isolates on *Gaeumannomyces graminis* var. tritici. All antagonist-pathogen combinations were examined on 10-15 mL of potato dextrose agar in 9 cm petri plate with four replicates per treatment. The plates were incubated 5 days for dual culture and 9 days for volatile metabolites and cell free culture at 25°C. The percent growth inhibition was calculated using the formula n = (a-b)/a×100, where n is the % growth inhibition; a is the colony area of uninhibited *Gaeumannomyces graminis* var *tritici*; and b is the colony area of treated *Ggt*.

Glasshouse test: The ability of *Pseudomonas* isolates to reduce the incidence and severity of take-all in wheat grown in glasshouse was investigated. Soil prepared by mixing of field soil, leaf compost, sand, organic manure by rate 1:2:2:2. Soil sterilized by autoclaving at 121°C for 30 min for 2 successive days. Inoculum of pathogen was prepared as follow. Wheat seed were soaked for 24 h in water at room temperature and then transferred to Erlenmyer flask and autoclaved for 15 min at 121°C on two successive days. *Ggt1* isolate was grown on potato dextrose agar (PDA) and when they were grown, piece of culture 5×10 mm in size were added to each Erlenmyer flask containing of autoclaved wheat, mixed with wheat and incubated at 20°C for 25 days. Wheat infested with pathogen was blended in SDW to make a slurry. The resultant inoculum were mixed by soil at ratio of 1% (w/w) (Etebarian *et al*., 2003). Bacterial isolates were grown in Potato Dextrose Broth (PDB) on a rotary shaker at 150 r-min^{-1} for 2 days at room temperature. Cell were pelletized by centrifugation at 2500 g for 10 min, suspended in 0.01 M phosphate buffer (pH = 7), repelletized, and resuspended in buffer and mixed with equal volume of 1% methylcellulose and added to disinfected seeds (sodium hypochloride 0.5% for 3 min) at 8 mL/100 g^{-1} seeds. The initial population on the seeds was determined by dilution-plating immediately following the treatment. The initial population of cell strains on the seed was about 10^9 colony per seed. Spring wheat cultivar Shiraz was used in this experiment. Triadimenol was used as effective fungicide for chemical control of take-all by seed treatment at rate of 0.2% (w/w). Treated seed were dried in laminar flow hood for 1 h. Ten seed were planted in 10 cm diameter pot (Mathre and Johnston, 1986; Milus and Rothrock, 1997).

Pots were watered at 2 days intervals until emergence and daily thereafter. There were four replicates per treatment, arranged in a randomized complete design with 16 treatments (Table 2). The experiment was carried out in a glasshouse at day temperature 32°C and night temperature 25°C with natural day light without supplementary lighting from September to October 2005 at Abourayhan Campus of Tehran University, Pakdasht, Iran. Plants were harvested 5 weeks after sowing and severity of diseases on roots was assessed using a 0-5 scale modified from Rothrock (1986). Root height, root fresh weight, root dry weight, shoot fresh weight and shoot dry weight are also determined.

Microplot trial: Microplot trial was conducted at Abourayhan Campus University of Tehran, Packdasht, Tehran Province from March to June 2005. Three pots (each, 20 cm diameter and 25 cm height) were used as a microplot. Seed treatment and preparing of inoculum were done by methods previously noted in glasshouse test. The amount of inoculum which was applied to potting mix 10 g infested wheat per kg of soil (Czaban *et al*., 2004). The field soil (loam soil with 49% sand, 36.8% silt, 14.2% clay) was mixed with organic manure at 2:1 rate (v/v). Approximately 60 seeds of spring wheat cultivar Shiraz were sown in each microplot. There were three replicates per treatment, arranged in randomized complete block design with 14 treatments (Table 3). Microplot were watered at 7 days intervals at early spring and 3 days intervals when the air temperature increased at late spring. Chemical control of aphids and leafhopper was carried out by insecticide and weed control was done mechanically.

All plants in any microplot harvested 14 weeks after sowing when plants given to ripening period. Observations were consisted of diseases rating (0-5) as mentioned above, percentage of blackended crowns, root fresh weight, root dry weight, shoot height and weight of 100 grain.

Statistical analysis: Data on percentage inhibition of growth of the pathogens *in vitro* were subjected to arcsin square root transformation before analysis. These data were analyzed using the MSTAT-C and means were compared by Duncan's Multiple-Range Test at $p \leq 0.05$. In harvesting process of microplot test, all plants in each microplot were harvested and the number of plant in each microplot calculated as a covariate. Therefore means of weighted observation corrected by software and covariance analysis exert for these data.

RESULTS

Effect of *Pseudomonas* isolates on mycelial growth on *Ggt*: Dual culture, volatile metabolite test and cell free culture test showed that all isolates of *Pseudomonas* tested inhibited growth of *Gaeumannomyces graminis* var. *tritici*. Inhibition varied from 32 to 73% in dual culture, from 52 to 96% in volatile metabolite test and from 77 to 98% in cell free culture test. Result of dual culture indicated the mycelial growth of *Ggt* was numerically reduced more by *Pseudomonas fluorescens* bioIII (21P) and *P. fluorescens* bioV (5 kM) than the other isolates tested. In volatile test, the reduction of mycelial growth of pathogen by *Pseudomonas fluorescens* bioIII (21P) was more than the other isolates. *P. fluorescens* bioV (32J) and *P. fluorescens* bioIII (5kM) were the best isolates in cell free culture which inhibited growth of the pathogen by 90% (Table 1).

Table 1: Effect of *pseudomonas* isolate on mycelial growth of *Ggt* as % of colony area compared to control

Treatments	Dual culture	Volatile metabolite	Cell free culture
P. fluorecens CHAO	32.5d	88.0b	81.9c
P. fluorecens bioIII (22P)	45.7c	52.2c	77.5c
P. fluorecens bioIII (21P)	60.1b	96.6a	86.0b
P. fluorecens bioV (5 kM)	73.2a	89.7b	98.3a
P. fluorecens bioV (32J)	56.8b	94.1a	98.3a

Significant differences are denoted by different letters within each column according to Duncan,s Multiple Range Test at $p \leq 0.05$.

Glasshouse test: The result of seed soaking with *Pseudomonas* isolates indicated that take-all was reduced by all *Pseudomonas* isolates tested. Both *Pseudomonas fluorescens* CHAO and *P. fluorescens* bioIII (21P) were more effective in reducing diseases index than other isolates. There were no significant difference between *Ggt* only and Triadimenol+*Ggt* treatments in diseases severity index. *Pseudomonas fluorescens* CHAO and *P. fluorescens* bioIII (21P) promoted root height, fresh and dry weight of roots and shoots compared to *Ggt* alone. There were no differences among *Ggt* only, *Ggt*+methylcelloluse and *Ggt*+triadimenol treatments in all observations (Table 2).

Microplot trial: All the Pseudomonads isolates significantly reduced diseases incidence of wheat take-all compared to *Ggt* only control. *Pseudomonas fluorescens* CHAO and *P. fluorescens* bioIII (21P) were more effective

Table 2: Effect of *Pseudomonas* isolates on *Ggt* in glasshouse condition

Treatments	Diseases rating (0-5)	Root height (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Healthy control	0e	15.25ab	7.20a	2.64a	31.14a	31.65b	5.14a
Healthy control+Methyl cellulose	0e	13.50b	7.33a	2.55a	32.05a	32.61ab	5.46a
P. fluorecens CHAO	0e	15.67ab	7.35a	2.75a	34.28a	35.80a	5.12a
P. fluorecens bioIII (22P)	0e	15.63ab	6.28a	2.76a	32.11a	35.73a	5.43a
P. fluorecens bioIII (21P)	0e	16.50a	7.06a	2.85a	33.56a	33.55ab	5.81a
P. fluorecens bioV (5 kM)	0e	15.38ab	6.44a	2.83a	32.70a	31.44b	5.73a
P. fluorecens bioV (32J)	0e	15.38ab	6.23a	2.83a	32.36a	31.87ab	5.60a
Fungicide(Triadimenol)	0e	14.00b	6.44a	2.80a	34.46a	30.09b	5.14a
Ggt only	4.90a	7.38d	3.23cd	1.06c	20.96b	18.48d	3.14c
Ggt+Methyl cellulose	4.60a	6.88d	3.00cd	1.06c	21.53b	19.76d	2.87c
Ggt+ *P. fluorecens* CHAO	1.90d	10.63c	4.49b	2.00b	23.86b	24.11c	4.15b
Ggt+ *P. fluorecens* bioIII (22P)	3.30c	7.00d	2.75cd	1.14c	23.84b	20.02d	2.91c
Ggt+ *P.* fluorecens bioIII (21P)	1.90d	10.63c	4.40b	2.05b	24.91b	25.99c	4.20b
Ggt+ *P. fluorecens* bioV (5kM)	4.40ab	6.88d	2.61cd	1.15c	23.66b	17.88d	2.89c
Ggt+ *P. fluorecens* bioV (32J)	4.00b	8.63cd	2.28d	1.38c	20.49b	18.45d	3.03c
Ggt+ Fungicide(Triadimenol)	4.90a	7.00d	3.50bc	1.15c	24.58b	16.45d	3.02c

Significant differences are denoted by deferent letters within each column according to Duncan,s Multiple Range Test at $p \leq 0.05$ and values are average of 4 replicates. *Ggt* = *Gaeumannomyces graminis* var *tritici*. Disease rating (0-5): 0 = root with no symptom, 1 = lesion on <25% of the root, 2 = lesion on 25% to <50% of the root, 3 = lesion on 50% to <75% of the root, 4 = lesion on 75% to 100% of the root , 5 = lesion on 100% root by blackended crown (Rothrock, 1986)

Table 3: Effect of *Pseudomonas* isolates on *Ggt* in microplot trial

Treatments	Disease rating (0-5)	Infected crowns (%)	Root fresh weight (g)	Root dry weight (g)	Shoot dry weight (g)	Weigh of 100 grain (g)
Control	0f	0e	79.12bc	18.00ab	39.31bc	2.45cd
Fungicide(Triadimenol)	0f	0e	66.47bc	15.03bc	32.50c	2.40d
P. fluorecens CHAO	0f	0e	107.5a	22.90a	52.84a	2.52abc
P. fluorecens bioIII (22P)	0f	0e	66.23cd	25.94bc	31.46c	2.53ab
P. fluorecens bioIII (21P)	0f	0e	91.17ab	17.04bc	43.31b	2.80a
P. fluorecens bioV (5 kM)	0f	0e	62.76cd	14.88bc	35.65bc	2.54ab
P. fluorecens bioV (32J)	0f	0e	63.53cd	19.65ab	33.48c	2.48bc
Ggt	4.50a	100a	13.57f	6.48e	8.35e	1.17h
Ggt+ Fungicide(Triadimenol)	2.20b	69.32b	30.26ef	9.69de	15.26de	1.34g
Ggt+ *P. fluorecens* CHAO	0.90e	44.96d	45.26de	14.22bcd	19.93d	2.02e
Ggt+ *P. fluorecens* bioIII (22P)	1.70c	71.36b	33.02f	8.28e	12.64de	1.55f
Ggt+ *P. fluorecens* bioIII (21P)	1.30d	60.03c	53.83d	11.83cde	20.81d	1.90e
Ggt+ *P. fluorecens* bioV (5 kM)	2.40b	63.63c	22.60f	7.18e	5.920e	1.17h
Ggt+ *P. fluorecens* bioV (32J)	2.30b	73.72b	22.55f	9.07e	8.452e	1.27g

Significant differences are denoted by different letters within each column according to Duncan,s Multiple Range Test at $p<0.05$. Percentage of infected crowns were subjected to arcsin square root transformation prior to analysis of variance. *Ggt* = *Gæumannomyces graminis* var. *tritici*. Disease rating (0-5): 0 = root with no symptom, 1 = lesion on <25% of the root, 2 = lesion on 25% to <50% of the root, 3 = lesion on 50% to <75% of the root, 4 = lesion on 75% to 100% of the root, 5 = lesion on 100% root by blackended crown (Rothrock, 1986)

in reducing take-all than the other isolates tested. The *Ggt*+fungicide treatment differ from *Ggt* only control and reduced disease severity and percentage of infected crowns. *Pseudomonas fluorescens* CHAO promotes fresh and dry weight of root and dry weight of shoot compared to *Ggt* only control.

Seed inoculation with *Pseudomonas fluorescens* bioIII (21P) significantly promoted root fresh weight, shoot dry weight, weight of hundred seed compared to pathogen control (Table 3).

DISCUSSION

All the *Pseudomonas* isolates reduce mycelial growth of pathogen by means dual culture, cell free culture, volatile metabolite test. The zone of inhibition on PDA suggest the synthesis of some antibiotic by the examined *Pseudomonas* isolates. Weller (1988) and Weller *et al.* (1997) claimed that the antibiotic production was one of the most important feature of bacterial with regard to take-all control on wheat. Phenazine-1-carboxylic acid (PCA) was the first such antibiotic to be identified but production of 2,4-diacetylphloroglucinol (DAPG) by fluorescent pseudomonads is now believed to be more important in suppressive soil than production of PCA (Weller *et al*., 1997; Ryder *et al*., 1990). Similar antibiotics for example pyoluteorin (Howell and Stipanovic, 1980), pyrrolnitrin (Howell and Stipanovic, 1979) noted for suppressive of pathogens by *Pseudomonas* strains. Other mechanism of diseases suppressing are volatile metabolites (especially HCN) and siderophores may involved in diseases suppression (Skaukat and Siddiqui, 2003).

It should be noted that the size of the inhibition zone is not a reliable indicator of inhibition strength because the inhibition zone is based the mobility of the antifungal compounds. This can be influenced by the polarity of the compound moving through the agar or the molecular size of the compound. Because of the complexity and number of antifungal metabolites that are produced by *P. fluorescens* strains (Etebarian *et al*., 2005), further research will be needed to identify and characterize the antifungal compounds produced by isolates tested.

Pseudomonas fluorescens CHAO showed lower percentage of mycelial growth inhibition, however in glass house and microplot study this isolate named the best isolates. The possible mechanism for suppression of take-all could be induced resistance in wheat plants due to root colonization by this strain (Bakker *et al*., 2003). *Pseudomonas fluorescens* bioIII (21P) inhibited growth of *Ggt in vitro* was also effective on glasshouse and microplot trials.

The result of glasshouse and field experiment indicated that, *P. fluorescens* CHAO and *Pseudomonas fluorescens* bioIII (21P) was effective in suppression of take-all in field and glasshouse condition. *P. fluorescens* CHAO also previously suppressed a variety of soil-borne plant pathogen (Weller *et al*., 1988).

Pseudomonas fluorescens bioIII (21P) which was isolated from rhizosphere of wheat inhibited teletospore germination of *T. leavis in vitro* and also controlled common bunt in glasshouse condition (Khodayegan, 2003), but the other isolates tested here had lower effect in glasshouse than microplot study. Microorganism population and physiochemical properties of soil may cause of take-all decline in our microplot study. The relative importance of antibiotics and siderophore in diseases suppression by a strain may depend on environmental conditions. Factor such as soil matric potential (Howei *et al*., 1987; Weller, 1988) and

rhizosphere pH influence the colonization of wheat roots by introduced bacteria and it is likely that the regulation of antibiotic and siderophore production would be even more sensitive to soil physical and chemical factors. Baker (1968) pointed out that soil pH has an indirect role in siderophore-mediated diseases suppression because pH affects the amount of iron available to plants and microorganism. Further studies on the effect of soil physical and chemical factors on diseases suppression by *Pseudomonas fluorescens* isolates tested are greatly needed. Induction of local or systemic resistance in wheat to pathogen following bacterial isolate root colonization is yet another possible explanation for suppression take-all (Bakker *et al*., 2003).

Result of glasshouse experiments showed that, *P. fluorescens* CHAO and *Pseudomonas fluorescens* bioIII (21P) when used as seed treatments alone increased root fresh and shoot dry weight compared with control without bacteria (Table 2), this is in accordance with results showing that some fungal isolates and bacterial strain are capable of promoting plant growth in glasshouse (Susloun, 1982; Salehpour *et al*., 2005). Plant growth promotion due to PGPR has been attribute to the increase of mineral nutrition available to root during the growth activity of PGPR isolates in the rhizosphere (Smith and Goodman, 1999).

Ryder *et al*. (1990), indicate that Pseudomonas strains was antagonistic to take-all and confirm our result. Capper and Higgins (1993) applied *Pseudomonas fluorescens* strain to wheat as a potential biological control agent against take-all. In their experiment *Pseudomonas fluorescens* 2-79 and *P. fluorescens* 13-79 reduce take-all severity under field condition. However, in our experiment *Pseudomonas* isolates caused lower effect in glasshouse than microplot study. With regard to that natural suppressive soil have been described for *Gaeumannomyces graminis* var. *tritici*, microorganism population and physiochemical properties of soil may should cause of take-all decline in our microplot study.

In the future, ability of bacterial strain to protect wheat against *Ggt* should be verified in field condition on different soil and with other strains of *Ggt*. Wong (1994) stated that the result of such field studies are inconsistent, with good response in some years and on some site but not on others and Mazzola *et al*. (1995) found that different strain of *Ggt* varied in their sensitivity (from very sensitive to insensitive) to antibiotic produced by fluorescens *Pseudomonas* spp.

In conclusion *Pseudomonas fluorescens* bioIII (21P) reduced disease severity in glasshouse and field condition. The isolate warrant further investigation for their ability to control take-all of wheat, especially in commercial situation and different environmental condition. An integrated approach using combination of biological agents and suitable fertilization may improve biological control of take-all of wheat.

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