Development of Formulations of *Pseudomonas fluorescens* for Control of Chickpea Wilt

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ABSTRACT

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Pseudomonas fluorescens strains were obtained from the rhizosphere of different crops and strains that showed inhibitory action against the chickpea (*Cicer arietinum*) wilt pathogen *Fusarium oxysporum* f. sp. *ciceris* were selected for further studies. The efficacy of various carriers in sustaining the population of these strains in storage was assessed. In talc-based and peat-based formulations the bacteria survived even up to 240 days of storage although the population declined from 30 days. When chickpea seeds were treated with talc-based formulations, *P. fluorescens* survived on the seeds for at least 180 days. When the treated seeds were sown in soil, the antagonist moved to the rhizosphere and survived well in it. Biopriming of seeds increased rhizosphere population. Seed treatment with the antagonist formulation effectively controlled chickpea wilt disease in the two field trials and increased the yield. When seed treatment was followed by root zone application the efficacy of *P. fluorescens* formulations increased. *Pseudomonas fluorescens* did not inhibit the beneficial N-fixing bacteria *Rhizobium* and *Azospirillum* in vitro and seed treatment fungicides (thiram and carbendazim) were not inhibitory to *P. fluorescens* in vitro.

Fusarium wilt caused by *Fusarium oxysporum* Schlechtend. f. sp. *ciceris* (Padwick) Matuo & Sato is a very serious disease in chickpea (*Cicer arietinum* L.) growing areas of the world but particularly in the United States, India, Spain, and in the Mediterranean region (3,24). The pathogen is soil-borne and drenching with fungicides is very expensive and impractical. Several strains of *Pseudomonas fluorescens* have been reported to suppress soil-borne diseases caused by fungal pathogens (15,29). Many greenhouse studies (10,11,14,23) and a few field experiments have been conducted (18,25) to show the efficacy of *P. fluorescens* in management of diseases. Commercial application of *P. fluorescens* for control of soil-borne diseases depends upon development of commercial formulations in which the bacteria can survive for a considerable length of time, development of suitable method of application to control early and late stages of disease development, and assessment of field efficacy of these formulations in the control of the disease as well as in increasing yield.

This paper reports selection of efficient *P. fluorescens* strains from rhizosphere, as well as development of formulations in which *P. fluorescens* survived for more than 8 months, controlled chickpea wilt under field conditions and increased yield. The practical utility of the formulation of *P. fluorescens* is discussed.

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MATERIALS AND METHODS

Isolation of fluorescent pseudomonads. Rhizosphere-rhizoplane colonizing *Pseudomonas* spp. were isolated from fresh roots of chickpea, pigeonpea, and rice plants from several geographic areas and soil types. After vigorous shaking of excised roots to remove all but tightly adhering soil, root segments (1 g) were shaken in 100 ml of sterile distilled water for 15 min. Fluorescent pseudomonads were isolated using King's medium B (KMB) (12).

Bioefficacy of fluorescent pseudomonads. To test the efficacy of the strains in inhibiting growth of *F. oxysporum* f. sp. *ciceris* in vitro, the bacteria were streaked at one side of the petri dish containing potato-dextrose agar (PDA) medium. Four-mm mycelial discs of 5-day-old *F. o.* f. sp. *ciceris* cultures in PDA were placed at the opposite side of the petri dish. The growth of the fungus toward the bacterial colony was suppressed, and the inhibition zone was measured. The bacterial strains which showed inhibition against *F. o.* f. sp. *ciceris* were identified by various tests specific for *P. fluorescens* (20).

Greenhouse studies. To test the efficacy of *P. fluorescens* strains in the control of chickpea wilt disease under greenhouse conditions, chickpea (cv. Shoba) seeds were sown in pots containing field soil into which was incorporated *F. o.* f. sp. *ciceris* cultures grown on sand-maize medium (19). Four seeds were sown per pot and 25 pots per strain were maintained. The fungal culture was incorporated in the ratio of 1:19 (sand-maize inoculum/soil). The seeds were treated with cell suspensions in water (10^9 cfu per ml) of the various *P. fluorescens* strains. The wilt incidence was assessed up to 90 days after sowing.

Survival of *Pseudomonas fluorescens* strains in different formulations. *Pseudomonas fluorescens* strains were grown on liquid KMB for 48 h as shake culture incubating in Infors AG shaker at 150 rpm at room temperature (25 ± 2°C). Bacterial survival was tested in six different carriers: peat, farmyard manure, talc, vermiculite, kaolinite, and lignite. Ten grams of carboxymethylcellulose was added to 1 kg of the carrier and mixed well. The pH of all materials was adjusted to 7.0 by adding calcium carbonate. The carriers were autoclaved for 30 min on each of two consecutive days. Four hundred milliliters of the bacterial suspension containing 9×10^8 colony-forming units (cfu) per ml was added to 1 kg of the carrier and mixed well under sterile conditions. The materials were packed in polythene bags, sealed, and incubated at room temperature (25 ± 2°C). Samples were drawn at intervals and the bacterial population was assessed using KMB by dilution plate method. Three independent samples were analyzed and there were three replications for each analysis. The experiment was set up as a randomized complete block design, 2 x 2 factorial replicated three times. Duncan's multiple range test (DMRT) was followed for the column factor and least significant difference (LSD) test for the row factor analysis (8). The package used for analysis was IRRI STAT version 92-1 developed by International Rice Research Institute Biometrics Unit, Philippines.

Survival of *P. fluorescens* on treated chickpea seeds. Chickpea seeds (cv. Shoba) were treated with talc-based formulations of three strains of *P. fluorescens* at 4 g per kg of seed. The talc-based formulation with 35% moisture content adhered well to the seed. Treated seeds were stored in polythene bags at room temperature. Survival of the bacteria on chickpea seeds was assessed at intervals by transferring 50 g of seeds to 100 ml of sterile water and dilution plating on KMB. Three independent samples with three replications each were analyzed. The experiment was set up as a factorial (8).

Establishment of *P. fluorescens* in rhizosphere. To assess the efficacy of *P. fluorescens* strains established in chickpea rhizosphere, one of the strains, Pf1, was developed as an antibiotic-resistant strain. During the preliminary screening, rifampicin at 190 µg per ml inhibited the growth

of Pf1 in KMB. Penicillin at 90 µg per ml, actinomycin D at 100 µg per ml, and streptomycin at 30 µg per ml inhibited growth of Pf1. The bacterium was grown in KMB with rifampicin (195 µg per ml); resistant colonies were selected and streaked onto KMB without antibiotic. From this, single colonies were selected and re-streaked onto rifampicin-supplemented medium. A marked strain was obtained by repeating the process with rifampicin-resistant strains cultured in sequence on KMB containing penicillium G (90 µg per ml), actinomycin D (100 µg per ml) and streptomycin 30 µg per ml. The marked strain was kindly supplied by R. Rabindran, Tamil Nadu Agricultural University. The marked strain that grew well in KMB containing all four antibiotics was used to assess its rhizosphere survival capability.

Chickpea seeds were treated with talc-based formulations of the marked Pf1 strain at 4 g per kg seed and shade dried for 2 h. Biopriming of treated seeds was done by incubating the treated seeds for 20 h at 25°C over sterile vermiculite moistened with sterile water in self-sealing plastic bags (6). Seeds (with or without biopriming) were sown in pots containing unsterilized soil. In another set of pots, seed treatment was followed by soil application of peat-based formulation at the rate of 2.5 kg formulation mixed with 25 kg farmyard manure (25 mg of the formulated product for four plants in a pot) per ha on 20 days after sowing. Four seeds were sown in each pot and 5 pots were kept as one replication. There were 3 replications and the pots were arranged in randomized block design. The Pf1 formulation was placed at the root region of the plants at about 5 to 10 cm depth manually. The rhizosphere population of Pf1 was assessed at intervals by the method described by Papavizas and Davey (16). One plant from each pot (5 plants per replication) was pulled out gently with roots intact. The root portion was cut and transferred to 100 ml of sterile water in an Erlenmeyer flask. After thorough shaking the population of Pf1 in the suspension was estimated by dilution plate method using KMB containing all four antibiotics. Fluorescent colonies were viewed under UV light. Five petri dishes for each dilution and three replications were maintained. The root samples were weighed and the population of the bacterium was expressed per g of root sample (dry weight).

Field trials. Two field trials with seven treatments and a control were conducted, one in the university experimental farm and another in a farmer's field. Chickpea (cv. Shoba) seeds previously treated with *Rhizobium japonicum* were treated with talc-formulation of three different strains of *P. fluorescens* and sown in the field. One of them, Pf1, is the marked strain. In another set of treatments, the peat-based formulations were applied in the field at 30 days after sowing at the rate of 2.5 kg per ha. The formulations were mixed with 25 kg of farmyard manure and manually placed in the 5 to 10 cm root zone of the plants. As a check, seeds were treated with carbendazim at 2 g kg^{-1} seeds; the fields were drenched with 0.1% carbendazim 30 days after sowing. In control plots, no seed or soil treatments were given with *P. fluorescens*. The plot size was 4 × 5 mm and 670 seeds were sown per plot at the spacing of 30 × 10 cm. The trial was laid out in randomized complete block design with three replications. Field emergence was assessed 15 days after sowing by counting the surviving plants in the entire plot. *Pseudomonas fluorescens* populations in the chickpea rhizosphere were assessed at intervals by pulling out 5 plants at random from each plot in each replication. Three independent samples with 5 replications (petri dishes) in each treatment were used for assessing the rhizosphere population. KMB was used to detect fluorescent pseudomonads. The wilt incidence in the entire plot was assessed and expressed as percentage of disease incidence. Arc sine transformation of data on percentage of field emergence and wilt incidence was done and DMRT was first applied to the transformed means and then transferred to the original means (8). At random the infected roots were plated on PDA and presence of the pathogen was assessed. Grain yield was also recorded and the yield data were analyzed by DMRT (8).

Compatibility of *P. fluorescens* with *Rhizobium japonicum*: In vitro test. To test the inhibitory action of *P. fluorescens* against *Rhizobium japonicum*, *P. fluorescens* strain Pf1 was streaked at one corner of the petri dish containing KMB. After 2 days of growth, *R. japonicum* was streaked perpendicularly to the *P. fluorescens* growth. After incubating for 5 days the inhibition zone was assessed.

In vivo test. Chickpea seeds were treated with talc-based *P. fluorescens* Pf1 (marked strain) at 4 g per kg of seed and then mixed with commercially available peat-based *Rhizobium japonicum* at 10 g per kg of seed adding 5 ml of 0.05% gum arabic and sown in pots containing about 5 kg of soil. Chickpea seeds treated with *P. fluorescens* alone or *R. japonicum* alone served as check. An untreated control was also maintained. Four seeds per pot, five pots per replication, and three replications for each treatment were maintained. The pots were arranged in randomized block design. The rhizosphere population of the marked *P. fluorescens* strain Pf1 was assessed after 60 days of plant growth by the methods already described. The number of nodules formed by *R. japonicum*, plant height and root length per plant were also assessed by uprooting all the plants after 60 days of growth.

Compatibility of *P. fluorescens* with *Azospirillum lipoferum*. To test the possible in vitro inhibitory action of *P. fluorescens* against *Azospirillum lipoferum*, another nitrogen-fixing bacterium, *A. lipoferum* was streaked perpendicular to the *P. fluorescens* growth on KMB as described for *R. japonicum*. After incubating for 5 days the inhibition zone was assessed.

Compatibility of *P. fluorescens* with seed treatment fungicides in vitro. Carbendazim and thiram are the most commonly used seed-treatment fungicides. The fungicides at various concentrations (100, 200, 500, 1,000, and 2,000 µg per ml) were incorporated in KMB. *Pseudomonas fluorescens* Pf1 was streaked on the medium. The bacteria streaked on KMB without any fungicide served as control. The bacterial growth on the streak was assessed after 5 days.

RESULTS

Twenty-seven fluorescent pseudomonad strains were tested for their efficacy in suppressing the growth of *F. o.* f. sp. *ciceris* in vitro. Four of them, viz., Pf1, Pf27, Pf2, and Pf21, were inhibitory in vitro to *F. o.* f. sp. *ciceris* with inhibition zones of 41, 41, 35, and 14 mm, respectively. The first three strains were statistically different from Pf21. Only these three strains significantly reduced disease incidence when tested under greenhouse conditions (20, 14, and 14% disease incidence in Pf1-, Pf27-, and Pf2-treated pots, respectively, compared with 60% cent incidence in both Pf21-treated and untreated control pots).

In all carrier formulations, *P. fluorescens* Pf1 survived up to 20 days without any dramatic decline from the initial population (Table 1). Although subsequently

Table 1. Survival of *Pseudomomas fluorescens* Pf1 in various carrier formulations[y]

Carrier formulation	Population (10^7 cfu per g) at various days of storage						
	0	10	20	30	40	120	240
Talc	37.5 a[z]	40.0 a	40.0 a	27.5 b	17.3 a	11.0 a	1.3 a
Vermiculite	30.0 ab	30.0 b	30.0 b	12.5 c	6.8 b	4.3 b	0.1 a
Kaolinite	30.0 ab	30.0 b	27.5 b	12.5 c	7.0 b	2.8 b	0.0 a
Lignite	30.0 ab	30.0 b	25.0 b	12.5 c	8.3 b	5.3 b	0.0 a
Peat	35.0 a	37.5 a	40.0 a	35.0 a	16.5 a	5.0 b	0.7 a
Farmyard manure	30.0 ab	30.0 b	28.0 b	15.0 c	8.0 b	3.5 b	0.1 a

[y] Similar results were obtained with *P. fluorescens* Pf2 and Pf27 strains.

[z] Mean of three replications. Means followed by a common letter in a column are not significantly different ($P = 0.05$) according to Duncan's multiple range test. Least significant difference (LSD) 0.05 value for comparing means in a row is 5.8.

there was a decline in the population, 120 days after incubation the bacterial population was 5 to 11 × 10^7 cfu per g in talc-based, peat-based, and lignite-based formulations. After 240 days of storage, also about 1 × 10^7 cfu were detected in talc- and peat-based formulations (Table 1). Similar results were obtained with the other two isolates, Pf2 and Pf27 (data not shown).

When talc formulations of the three strains of *P. fluorescens* were applied to chickpea seeds, the bacteria survived well throughout the experimental period of 180 days in storage (data not shown). When the seeds were sown in pots under greenhouse conditions, the bacteria could be detected in the rhizosphere. When the treated seeds were given biopriming treatment the population in the rhizosphere was significantly higher than those without biopriming (Table 2). Soil application also increased the rhizosphere population and the increase was on a par with biopriming treatment.

In the field trials, among the three strains used only Pf1 was the marked strain. Hence, KMB without any antibiotics was used to detect general fluorescent pseudomonad populations in chickpea rhizosphere. Seed treatment with *P. fluorescens* strains resulted in increased population of fluorescent pseudomonads in the rhizosphere. Maximum populations were observed 60 days after sowing. Root zone application of *P. fluorescens* resulted in further increase in rhizosphere population of fluorescent pseudomonads (Table 3).

Application of *P. fluorescens* increased field emergence of chickpea plants. Wilt incidence was observed even at 15 days after sowing (Table 4). In the first trial conducted at the university farm, the disease incidence increased along with increase in the age of the crop. But in the second trial conducted at a farmer's holding severe disease incidence was observed at 15 days after sowing itself. The disease was effectively controlled by *P. fluorescens* treatment. Effectiveness of *P. fluorescens* increased if it was applied in root zone of the plants at 30 days after sowing, besides as seed treatment. *Fusarium oxysporum* f. sp. *ciceris* was invariably isolated from the wilted plants. But in some instances *Rhizoctonia bataticola* could also be isolated along with *F. o.* f. sp. *ciceris*.

Pseudomonas fluorescens treatments, particularly seed treatment followed by root zone application, increased the yield significantly (Table 4). In one of the trials, the yield from *P. fluorescens* treatment (seed + root zone applications) was much higher than the yield after carbendazim application itself, while in the other trial it was on a par with the fungicide application.

Pseudomonas fluorescens did not inhibit *R. japonicum* or *A. lipoferum* in vitro. It also did not affect nodulation induced

Table. 2. Survival of *Pseudomonas fluorescens* marked strain Pf1 in chickpea rhizosphere under greenhouse conditions

Treatment	*Pseudomonas fluorescens* population (10^4 cfu per g) in rhizosphere		
	30	45	60
Seed treatment[w]	4.8 b[x]	7.2 b	8.1 b
Biopriming[y]	9.4 a	12.3 a	15.4 a
Seed treatment + soil application[z]	9.8 a	13.8 a	16.5 a
Control	0.0 c	0.0 c	0.0 c

[w] Seed treatment with *P. fluorescens* was done at the time of sowing.
[x] Mean of three replications. Means followed by a common letter in a column are not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[y] Biopriming of treated seed was done by incubating the treated seeds for 20 h at 25°C over sterile vermiculite moistened with sterile water in self-sealing plastic bags.
[z] Seed treatment with *P. fluorescens* at the time of sowing was followed by soil application of it 20 days after sowing.

Table 3. Fluorescent pseudomonad population in chickpea rhizosphere under field conditions

Treatment[y]	*Pseudomonas fluorescens* strain	Fluorescent pseudomonad population (10^4 cfu per g) in rhizosphere at different days after sowing					
		Trial I			Trial II		
		15	45	60	15	45	60
Seed treatment alone	Pf1	3.0 a[z]	9.0 d	12.0 c	4.0 a	8.0 e	10.0 e
	Pf2	3.3 a	9.6 d	13.0 c	3.7 a	9.6 d	11.3 e
	Pf27	4.0 a	8.6 d	12.0 c	3.0 a	11.0 c	15.0 d
Seed treatment + soil application	Pf1	3.3 a	16.3 b	20.0 b	3.0 a	15.3 b	19.0 b
	Pf2	3.0 a	15.6 c	19.3 b	3.3 a	14.3 b	17.3 c
	Pf27	3.3 a	17.3 a	21.3 a	4.3 a	19.0 a	23.0 a
Carbendazim (check)		1.0 b	2.6 e	3.3 d	1.3 b	2.7 f	3.0 f
Control		1.0 b	2.6 e	3.6 d	1.3 b	3.3 f	3.7 f

[y] *Pseudomonas fluorescens* strains were applied either as seed treatment alone or seed treatment at the time of sowing followed by soil application 30 days after sowing. Carbendazim was applied as seed treatment and the fields were drenched with it 30 days after sowing as check.
[z] Means followed by a common letter in a column are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table. 4. Efficacy of *Pseudomonas fluorescens* (Pf1) strains in management of chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris*

Treatment[y]	*P. fluorescens* strain	Field emergence (%)		Wilt incidence (%) at different days after sowing				Yield (kg per ha)	
				15		75			
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Seed treatment alone	Pf1	98.4 a[z]	98.9 a	2.4 b	3.1 b	15.9 b	17.9 b	992 b	1,167 d
	Pf2	98.2 a	98.5 a	2.4 b	2.5 b	16.7 b	16.8 b	894 c	1,134 d
	Pf27	99.1 a	99.1 a	1.8 b	1.6 b	15.5 b	18.3 b	945 b	1,050 e
Seed treatment + soil application	Pf1	100.0 a	99.5 a	0.2 bc	0.5 b	7.5 c	4.1 c	1,396 a	1,667 a
	Pf2	98.5 a	100.0 a	2.1 b	0.0 b	4.5 cd	5.5 c	1,395 a	1,583 b
	Pf27	100.0 a	100.0 a	0.0 bc	0.3 b	5.6 c	5.6 c	1,442 a	1,475 c
Carbendazim (check)	...	99.7 a	99.4 a	1.1 b	1.6 b	6.2 c	5.1 c	1,420 a	1,083 de
Control	...	79.3 b	63.1 b	26.7 a	40.7 a	42.8 a	50.2 a	700 d	700 f

[y] Arc sine transformation of the data was done prior to analysis. Duncan's multiple range test was first applied to the transformed means and then transferred to the original means. Means followed by a common letter in a column are not significantly different ($P = 0.05$).
[z] *Pseudomonas fluorescens* strains were applied either as seed treatment alone at the time of sowing or as seed treatment at the time of sowing followed by soil application 30 days after sowing. Carbendazim was applied as seed treatment and the fields were drenched with it 30 days after sowing as check.

by *R. japonicum*. Number of nodules in plots treated with *R. japonicum* alone was 25.0 per plant while it was 25.3 in plots treated with *R. japonicum* + *P. fluorescens*; these numbers were not statistically different from each other. In the plots without *R. japonicum,* 9.3 nodules per plant were observed. Neither carbendazim nor thiram, even at 2,000 µg per ml, were inhibitory to *P. fluorescens* in vitro (data not shown).

DISCUSSION

Seed treatment with cell suspensions of *P. fluorescens* has been found effective in controlling several diseases (11,18,25, 27,30). For commercial exploitation, this methodology will be impractical due to difficulty in handling, transport, and storage. In the present studies, we have shown that the bacteria can survive well in talc- or peat-based formulations for more than 8 months. Plant-growth-promoting rhizobacteria (PGPR) have been reported to survive in certain dry formulations (21). Populations of PGPR did not decline in the talc mixture with 20% xanthan gum after storage for 2 months at 4°C (13).

Survival of *P. fluorescens* in seeds coated with the talc-based formulations would allow the antagonist to be supplied to the farmers as either a talc-based formulation for seed treatment or to the seed producer to supply treated seeds to farmers. When the treated seeds were sown the bacteria established well in the rhizosphere. For the effective management of any soil-borne disease, the introduced antagonist should colonize roots (28). It is difficult to introduce an antagonist to the established rhizosphere since once a portion of root is occupied by the native bacteria, the introduced bacteria are unable to displace them (27). The successful antagonist should colonize the rhizosphere at the time of seed germination itself; in other words, the antagonist should move from spermosphere to rhizosphere and establish there (27). The movement of *P. fluorescens* from seed to rhizosphere of chickpea is indicated by our studies on the effect of biopriming. Biopriming is known to increase the population of *P. fluorescens* more than 10-fold in seed (6) and this was reflected in an increase in the population of *P. fluorescens* in the rhizosphere of bioprimed seeds.

Root zone application of peat-based *P. fluorescens* formulation increased rhizosphere population of the bacteria. Certain strains of fluorescent pseudomonads have been shown to provide biological control of root pathogens when applied to soil (4) since some of these strains have the ability to colonize the roots (17,29). The establishment of *P. fluorescens* in the rhizosphere of plants is due to an improved capacity to compete for root exudates (7) and disease suppression by the introduced bacteria depends on their ability to colonize roots (27,28).

The field emergence of chickpea plants was improved by seed treatment with a talc-based *P. fluorescens* formulation. This may be because the formulation suppresses pre-emergence damping off caused by various pathogens. Several pathogens are known to suppress the field emergence of chickpea plants (11,25). A combination of seed treatment and root zone application effectively controlled chickpea wilt. Chickpea show wilting at different growth stages. Earlier wilting is followed by severe wilting at the flowering stage (1). Root zone application of the bacterial formulation would have reduced late wilting effectively. Soil application of the antagonists is also effective in the control of various diseases (15) but the major problem is the lack of a delivery system that is economically and logistically practical. The peat-based formulation may be ideal for placing the bacteria in the root zone. Peat-based formulations are widely used to introduce *Azospirillum* spp., a biofertilizer, into the rhizosphere (9).

Treatment with *P. fluorescens* formulations increased chickpea yield in both field trials. The yield increase is more than 100% over the untreated control, particularly in trial 2, in which the bacterial treatments resulted in much higher yields than did the fungicide–soil drenching treatment. *Pseudomonas fluorescens* is known to be a growth promoter and yield increases due to this bacterium have been reported in several crops (5,11,18,25,30).

Our studies have established that *P. fluorescens* formulations can be effectively used as seed treatments to control chickpea wilt. Nitrogen-fixing bacteria like *Rhizobium* and *Azospirillum* are also used as seed treatment (9,26) to increase the yield of chickpea crop. *Pseudomonas fluorescens* was not inhibitory to *Rhizobium or Azospirillum.* All three bacteria are rhizosphere bacteria and migrate toward roots in the soil (2). The noncompetitive nature of *P. fluorescens* vis-à-vis the other two bacteria is an additional advantage. Thiram and carbendazim are commonly used for seed treatment to increase the field emergence of chickpea and both the seed treatment fungicides were noninhibitory to *P. fluorescens.* Several fungicides have been reported to be noninhibitory to *P. fluorescens* (5,22).

Our studies have shown that chickpea wilt disease can be effectively controlled by *P. fluorescens* formulations that can be commercially exploited.

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