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Research Article

Combined application of *Pseudomonas fluorescens* and *Purpureocillium lilacinum* liquid formulations to manage *Globodera* spp on potato

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Abstract: Potato cyst nematodes (PCN), *Globodera rostochiensis* and *Globodera pallida* are major limiting factors to potato cultivation globally. Effective use of nematode antagonistic bio-agents is a potentially important component of the eco-friendly agro-farming. *Pseudomonas fluorescens* and *Purpureocillium lilacinum* are known for their nematode antagonistic potential and plant growth promotion ability. The effect of seed treatment with liquid suspension of *P. fluorescens* at 1 l/ton seeds and soil drenching with suspension of *P. lilacinum* at 5 l/ha, singly and jointly, was studied to minimize the damage caused by PCN in potato plants under field conditions in two regions in India. Both applications showed significantly greater PCN suppression and better plant growth promotion in comparison to solo application. The both application showed the highest reduction of cyst population (75.7%) in soil, female population (79.9%) in root and egg numbers per soil of each location (84%). The potato plants from *P. fluorescens*-seed treatment and *P. lilacinum*-soil drenching both applied plots were 33.5% taller with 45.6% more number of tubers than untreated plants. The tuber yield was also significantly higher (35.9%) in both application than untreated control. There was no significant difference on the root colonization of *P. fluorescens* and *P. lilacinum* in solo and both treatments.

Keywords: Potato cyst nematode, biological control, liquid formulations, *Pseudomonas fluorescens, Purpureocillium lilacinum*

Introduction

Potato *Solanum tuberosum* L. is an important tuber crop that is grown globally to meet food requirement of people in many countries. It is considered fourth important food crop after rice, wheat and maize. It is also used as animal feed and to make commercial starch products. In India, potato is cultivated in 28 states with total area of 20.24 million ha, producing 46.4 million tonnes annually (Welfare, 2016). Among the various pest and diseases associated with this crop, potato cyst nematodes, *Globodera rostochiensis* (Woll) and *Globodera pallida* (Stone) remain a daunting challenge for potato production. PCN are sedentary root endo-parasites. The second-stage juveniles (J2) penetrate through growing tips of roots and form feeding sites or syncytia in vascular tissues which lead to stunted growth, early senescence, proliferation of lateral roots and partial or complete arrest of tuber formation (Devrajan *et al*., 2004). In addition, root damage caused by PCN provides an avenue for

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entry of fungal pathogen such as *Rhizactonia solani* resulting in crop loss due to the synergistic disease complex (Back *et al*., 2006). In Europe and North America, the yield loss due to PCN has been reported as 9-100% (Pineda *et al*., 1993). In India, up to 80% yield loss due to PCN was reported from Nilgiris and Kodaikanal hills, Tamil Nadu region (Devrajan *et al*., 2011), Karnataka, Kerala and Himachal Pradesh (Krishna Prasad and Singh, 1986; Ramana and Mohandas, 1988; Sudershan *et al*., 2010).

The chemical nematicide carbofuran 3G is frequently used to control nematodes, but its repeated use is required to maintain cyst populations below the damage threshold levels (Seenivasan, 2017). The drawbacks of chemical nematicides such as the potential residue, groundwater contamination, enhanced biodegradation and toxicity to applicators also necessitated to search for alternative method of control. Biological control with fungal or bacterial organism that effectively antagonise the nematodes is an ecologically sound approach that has tremendous prospective to control nematode population build up and thereby reduce the crop damage (Seenivasan and Sundarababu, 2007). The root colonizing plant growth promoting rhizobacteria like *P. fluoresecens* have shown better result for the management of various plant parasitic nematodes such as *Hirschmanniella oryza* on rice (Seenivasn and Lakshmanan, 2002), *Globodera rostochiensis (*Devrajan *et al*., 2004), *Meloidogyne graminicola* on rice (Seenivasan, 2011), *Radopholus similis* on banana (Seenivasan *et al*., 2013), *Meloidogyne javanica* on tomato (Siddiqui and Shaukat, 2004), *Meloidogyne incognita* on medicinal coleus (Seenivasan and Devrajan, 2008) and jasmine (Seenivasan and Poornima, 2010). The facultative egg parasitic fungus, *Purpureocillium lilacinum* (= *Paecilomyces lilacinus*) has been reported to be effective against *Meloidogyne* spp and many other plant parasitic nematodes in various crops (Rao, 2008; Rao *et al*., 2012; Crow, 2013; Mohd *et al*., 2009). Earlier reports by Devrajan *et al*. (2004) and Seenivasan *et al*. (2007) demonstrated the biocontrol potential of *P. fluoresecens* and *P. lilacinum* against PCN in potato. In the field situation, the performance of bio-control agents is not efficient enough to provide sufficient nematode control as like that of chemical nematicides. Recently, the concept of combined use of different biocontrol agents was attempted on crops of tomato, pumpkin, sugar beet and chickpea and demonstrated successfully against various plant parasitic nematodes (Seenivasan *et al*., 2012). However, there are no reports on the combined use of these biocontrol agents for the management of PCN on potato. Hence this study aimed to find out the effect of combined use of liquid suspensions of *P. fluoresecens* and *P. lilacinum* to manage PCN in the field conditions.

Materials and Methods

Bio-formulations

The liquid formulation of *P. fluorescens* strain Pf1 containing 5×10^9 colony forming units (cfu)/ml was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. The liquid formulation of *P. liliacinum* strain Ooty1 containing 4×10^9 cfu/ml was obtained from Horticultural Research Station, Tamil Nadu Agricultural University, Ooty, India.

Field studies

Two field trials were conducted in the farmer fields naturally infested with PCN populations at Shenbaganur village (Location I) and Bambarpuram village (Location II), Kodaikanal, Tamil Nadu, India. The mixed populations of *G. rostochiensis* and *G. pallida* existed in both fields. Both trials were laid at similar time period from February 2016 to April 2016. Seed tubers of the potato cv. Kufri Jothi were used for field trials. Both experiments consisted of the following 5 treatments: (1) Seed treatment (ST) with liquid suspension of *P. fluorescens* (5×10^9 colony forming units (cfu)/ml) at 1 l/ha seed; (2) Soil drenching (SD) with liquid suspension of *P. lilacinum* (5×10^9

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colony forming units (cfu)/ml) at 5 l/ha; (3) ST with liquid *P. fluorescens* at 1 l/ton seed + SD with liquid *P. lilacinum* at 5 l/ha; (4) carbofuran 3G (Furadan 3G) at 1 kg a.i/ha; and (5) Untreated control. The experiments were laid out in randomized block design with five replications. The individual plot size was 3 × 5 m. For seed treatment, 1 l of *P. fluorescens* suspension was mixed with 50 l water + 250ml Tween 20 (sticking agent) in 100 l capacity plastic drums. The seeds were soaked in the respective suspensions for 15 min and immediately used for sowing. Seeds were sown leaving 30 cm space between each plants with 60cm space between rows. A total of 60 plants/plot was maintained. Each plot was separated by raised bunds leaving 0.5 m space between each bund. Soil drenching of liquid *P. lilacinum* was carried out immediately after sowing. The 5 l of *P. lilacinum* was diluted with 100 l water and applied in rows at 1 l/m in each plot. Standard agronomic practices for potato cultivation were followed for raising the crop.

General observations

The stem length was measured at 90 days after sowing (DAS) from randomly selected five plants per plot. Plants were harvested on 120 DAS and root tuber yield recorded from all plots. Yield was expressed in tonne (t) per ha. Number of tubers/plant was recorded from five randomly selected plants. The population density of cyst in soil from each plot was determined before treatment and at harvest. Each sample comprising of 10 random cores collected at a depth of 15-20cm and pooled together into a composite sample. A sub-sample of 200 cm^3 from each composite sample was processed by Fenwick's floatation method (Fenwick, 1940). The population of PCN cysts was counted under a stereoscope microscope. A subsample of 100 g soil was taken from each composite sample after thorough mixing and used for egg estimation. The cysts were extracted from the samples first by Cobb's sieving and decanting method (Cobb, 1918). The residue containing cysts collected from the 60 mesh (250µ) were crushed by mechanical cyst crusher to release eggs and the macerated suspension was poured through 625 mesh (20µm). Then the residue collected was processed by centrifugal floatation technique to separate eggs (Barker and Niblack, 1990). Eggs and juveniles were counted by viewing under a stereo zoom microscope (Kozo Zoom 645) at a magnification of 40x. Since, the juvenile population was very low in each plot and location, juvenile count was added with egg count. Five plants from each plot just before harvest were collected and female population per 2.5 cm root length were recorded under a stereo zoom microscope.

Re-isolation of introduced bio-agents

Root colonization of the introduced *P. fluoresecens* and *P. lilacinum* was assessed from 1 g root samples from each plot following the serial dilution plate technique as described by Seenivasan (2011). Kings B media and potato dextrose agar media were used for *P. fluoresecens* and *P. lilacinum,* respectively. Percentage of parasitized cysts by *P. lilacinum* was also assessed. Ten cysts were hand-picked, rinsed with sterile distilled water two times and plated on potato dextrose agar media in 90 mm Petri plates. The plates were incubated at 28 ± 3 °C for 15 days and fungi parasitization were observed under stereo zoom microscope. The percentage of parasitized eggs was calculated using the formula: (no of cysts infected with fungus/total number of cysts) × 100.

Statistical procedure

The data collected were analyzed for one-way analysis of variance using SPSS 16.0 for Windows software (SPSS Inc., Chicago, IL, USA). The treatment means were compared by Duncan's multiple range test (DMRT) (Panse and Sukhatme, 1954).

Results

Results showed that PCN cyst density and egg numbers in soil, as well as adult female

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population in roots were significantly reduced in *P. fluoresecens* and *P. lilacinum* treated plots in both fields (Tables 1 and 2). Combined application of seed treatment with *P. fluoresecens* and soil drench with *P. lilacinum* wasthe most effective in controlling PCN. This treatment reduced cyst population in soil by 75.5% in Location I and 75.8% in Location II, being significantly superior to their individual applications and also standard chemical carbofuran treatment. The *P. fluoresecens* seed treatment and *P. lilacinum* soil drench individually resulted in the smallest reduction of cyst populations (48.3% and 51.4%, respectively) over the control. The number of females/2.5 cm root was also significantly less in *P. fluoresecens* ST + *P. lilacinum* SD as compared to the individual treatments, carbofuran and untreated plants. The combined treatment reduced the root penetration of PCN by 79.8% compared to when *P. fluoresecens* ST and *P. lilacinum* SD were applied individually.

The number of PCN eggs in soil was significantly higher in control plots (Tables 1 and 2). The egg population in soil from all other treatment plots were found to be less than control. The plots treated with combination of *P. fluoresecens* ST + *P. lilacinum* SD had significantly least egg population that was 84% less than control plots. However egg population reduction was only 57.3% in *P. fluoresecens* ST and 55.1% in *P. lilacinum* SD treatments.

Plants from untreated plots were smaller and had fewer number of tubers compared to treated plots in both trials (Tables 1 and 2). Seed treatment with *P. fluorescens* accompanied with soil drench with *P. lilacinum* had significantly higher effect on plant growth improvement than all other treatments. The plants in this treatment were 33.5% taller with 45.6% more number of tubers than the untreated plants. The growth improvement was lesser in *P. lilacinum* SD treated plots than *P. fluorescens* ST alone and combination of *P. fluorescens* ST and *P. lilacinum* SD plots. The improved plant growth in carbofuran, *P. fluorescens* ST, *P. lilacinum* SD and combination of *P. fluorescens* ST and *P. lilacinum* SD plots resulted in significant increase in potato yield. The maximum tuber yield increase (35.9%) was noticed in the plots treated with combination of *P. fluorescens* ST and *P. lilacinum* SD followed by carbofuran (21.1%), *P. fluorescens* ST alone (16.1%) and *P. lilacinum* SD alone (15.5%) treated plots.

The introduced *P. fluorescens* and *P. lilacinum* singly or in combination survived in potato roots up to harvest. The colonization of roots by *P. fluorescens* was statistically uniform in plots applied individually or in combination with *P. lilacinum*. Similarly, root colonization and cyst parasitization by *P. lilacinum* were not significantly different compared with combination of *P. fluorescens* (Tables 1 and 2).

Table 1 Effect of liquid bio-formulation treatments on potato cyst nematode infection, growth and yield of potato cv. Kufri Jothi. (Location I).

Treatments	Cyst population /200 cm^3 soil		Eggs/ 100 g soil		Number of females /2.5 cm root	Plant height (cm)	Number of tubers/ plant	Tuber yield (t/ha)	Root colonization (CFU x 10^5 g^{-1} root)		Cyst parasitisation by *P. lilacinum* (%)
	Before treatment	120 DAT	Before treatment	120 DAT					*P. fluorescens*	*P. lilacinum*	
T_1	131 a	238 b	1902 a	5863 b	13.1 b	55.3 b	14.2 b	13.4 b	2065 a	-	-
T_2	128 a	224 b	1856 a	5623 b	12.4 b	54.1 b	14.9 b	13.9 b	-	197 a	58 a
T_3 (T_1 + T_2)	127 a	112 c	1894 a	1989 c	4.3 c	58.3 a	18.7 a	18.1 a	2012 a	192 a	53 a
T_4	125 a	208 b	1924 a	5148 b	11.6 b	54.9 b	15.2 b	14.7 b	-	-	-
UC	130 a	459 a	1863 a	12617 a	21.7 a	38.7 c	10.2 c	11.4 c	-	-	-

T1: Seed treatment with liquid *P. fluorescens* (1 L/ton seed), T2: Soil drenching with liquid *P. lilacinum* (5 L/ha), T4: Carbofuran 3G (1kg a.i/ha), UC: Untreated control. DAT: Days after treatment.

Means followed by the same letter in columns are not significantly different at P □ 0.05 according to Duncan's multiple rang test; CFU: colony forming units.

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Table 2. Effect of liquid bio-formulation treatments on potato cyst nematode infection, growth and yield of potato cv. Kufri Jothi. (Location II).

Treatments	Cyst population /200 cm^3 soil		Eggs/ 100 g soil		Number of females /2.5 cm root	Plant height (cm)	Number of tubers/ plant	Tuber yield (t/ha)	Root colonization (CFU x 10^5 g^{-1} root)		Cyst parasitisation by *P. lilacinum* (%)
	Before treatment	120 DAT	Before treatment	120 DAT					*P. fluorescens*	*P. lilacinum*	
T1	154 a	273 b	201 a	6318 b	15.2 b	56.0 b	13.7 b	12.8b	3153 a	-	-
T2	162 a	257 b	197 a	6072b	14.5 b	55.1 b	14.3 b	13.3 b	-	258 a	64 a
T3	152 a	128 c	192 a	2148 c	5.1 c	58.9 a	18.1 a	17.5 a	3117 a	243 a	60 a
(T1 + T2)	161 a	240 b	206 a	5556 b	13.7 b	55.9 b	15.7 b	14.2 b	-	-	-
T4	156 a	531 a	198 a	13260 a	25.0 a	39.2 c	9.8 c	11. 0 c	-	-	-

T1: Seed treatment with liquid *P. fluorescens* (1 L/ton seed), T2: Soil drenching with liquid *P. lilacinum* (5 L/ha), T4: Carbofuran 3G (1kg a.i/ha), UC: Untreated control, DAT: Days after treatment.
Means followed by the same letter in columns are not significantly different at P □ 0.05 according to Duncan's multiple rang test; CFU: colony forming units.

Discussion

The individual lethal effect of *P. fluorescens* and *P. lilacinum* have been demonstrated against *Meloidogyne* spp. (Seenivasan and Devrajan, 2008; Seenivasan and Poornima, 2010). In this study, the efficacy of PCN control was significantly higher by the combined application of *P. fluorescens* seed treatment and *P. lilacinum* soil drench than when they were used alone. Increasing population of biocontrol agents with antagonistic activities against nematodes in the rhizosphere have been reported to improve soil suppressiveness (Shaukat and Siddiqui, 2001). Similarly, control of *M. incognita* on tomato and bell pepper has been improved by combining *P. fluorescens* and *P. lilacinum* (Rao *et al*., 2012; Hashem and Abo-Elyousr, 2011). Seenivasan (2010) also showed that integration of *P. fluorescens* and *P. lilacinum* effectively reduced *M. incognita* and *Macrophomina phaseolina* disease complex on medicinal coleus. In addition, the synergistic effect between *P. fluorescens* and *P. lilacinum* was reported to provide more efficient and consistent nematode control in gladiolus fields (Sowmya and Rao, 2013). This study confirms that combined application of *P. fluorescens and P. lilacinum* is beneficial in the management of PCN.

The mechanism of PCN protection by *P. fluorescens* and *P. lilacinum* is attributed to the following direct or indirect effects. The root colonization by *P. fluorescens* has been reported to alter the root exudates that affect the nematode egg hatching, attraction towards root and root penetration potential (Seenivasan and Lakshmanan, 2002). In their study culture filtrates of *P. fluorescens* strain Pf1 was reported to have nematotoxic principle (Seenivasan and Lakshmanan, 2001). The *P. fluorescens* strain Pf1 also has the ability to induce systemic resistance against nematodes in plants by producing peroxidases, polyphenol oxidases, phenylalanine ammonia lyase and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzymes (Seenivasan, 2011; Saravanakumar and Samiyappan, 2006). It is a well-established fact that *P. lilacinum* colonizes roots of diverse plants, parasitizes cysts, eggs, juveniles and adult females of *Globodera* spp. by direct hyphal penetration (Jatala, 1986). Apart from direct parasitism, the development of *P. lilacinum* early in the soil might prevent the initial infection resulting in lower level of root penetration. All strains of *P. lilacinum* are reported to produce acetic acid and some metabolites like paecilotoxins and leucinostatins which are found to have detrimental effect on nematode juveniles (Singh *et al*., 2013). These metabolites may also probably be involved in reduction of PCN juveniles. Furthermore, being a parasite of mature females it would affect their egg production (Jatala, 1986) and *P. lilacinum* is capable of arresting syncytia formation induced by nematodes in plants (Cabanillas *et al*., 1988).

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The results proved that the *P. fluorescens* and *P. lilacinum* enhanced the growth of plants in addition to PCN reduction. Apart from their effect on nematode, *P. fluorescens* and *P. lilacinum* are recognized to possess plant growth promoting effect in many crop plants like pigeon pea and tomato (Siddiqui *et al*., 1998; Khan and Akram, 2000). The *P. fluorescens* strain Pf1 is reported to induce plant growth by producing plant growth regulators like indole acetic acid, gibberellins and cytokinins (Seenivasan, 2011). *P. lillacinus* on the other hand improves plant growth by increasing the available phosphorus in the soil (Lima-Rivera *et al*., 2016). The results of this study showed that root colonization of *P. fluorescens* did not affect *P. lilacinum* and vice-versa. Similar result was reported in bell pepper in which root colonization by *P. fluorescens* did not affect *P. lilacinum* (Rao *et al*., 2012).

It is concluded that seed treatment with *P. fluorescens* followed by *P. lilacinum* soil drenching can be recommended for the practical management of *G. rostochiensis* and *G. pallida* infection in potato fields rather than application of a single bio-agent.

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Disclosure statement

No potential conflict of interest was reported by the author.

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کاربرد ترکیبی از فرمولاسیون مایع باکتری *Pseudomonas fluorescens* و قارچ *Purpureocillium lilacinum* در مدیریت نماتدهای سیستی سیب‌زمینی *Globodera* spp

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چکیده: تماتدهای سیستی سیب‌زمینی شامل *Globodera rostochiensis* و*Globodera pallida* از عوامل اصلی محدودکننده تولید سیب‌زمینی در جهان محسوب می‌شوند. استفاده مؤثر از عوامل زیستی (بیوکنترل) بخش مهمی از کشاورزی دوستدار محیط‌زیست می‌باشد. خاصیت آنتاگونیستی باکتری *Pseudomonas fluorescens* و قارچ *Purpureocillium lilacinum* بر نماتدها و توانایی‌شان در بهبود رشد گیاهان شناخته شده است. در این پژوهش برای به حداقل رساندن خسارت ناشی از نماتد سیستی سیب‌زمینی، تیمار بذرها با سوسپانسیون *P. fluorescens* به میزان یک لیتر برای هزار کیلوگرم غده و خیساندن خاک با سوسپانسیون *P. lilacinum* به میزان ۵ لیتر در هکتار هر یک به‌تنهایی و هر دو با هم در دو منطقه در شرایط مزرعه در هندوستان مطالعه شد. نتایج نشان داد که در کاربرد ترکیبی از هر دو عامل بیوکنترل، سرکوب نماتد و افزایش رشد گیاه به‌طرز چشم‌گیری بیش‌تر از استعمال هر تیمار به تنهایی بود. در هر کدام از دو منطقه مورد آزمایش بیش‌ترین کاهش جمعیت سیست موجود در خاک (۷۵/۷ درصد)، جمعیت نماتد ماده در ریشه (۷۹/۹ درصد) و تعداد تخم در خاک (۸۴ درصد) در تیمار مرکب هر دو عامل بیوکنترل مشاهده شد. در هر دو قطعه زمین آزمایشی ارتفاع گیاهان و تعداد غده‌های سیب‌زمینی تیمار شده با هر دو عامل (بذر با*P. fluorescens* و خیساندن خاک با *P. lilacinum*) به‌ترتیب ۳۳/۵ درصد و ۴۵/۸ درصد بیش‌تر از گیاهان شاهد تیمار نشده بود. کلنیزاسیون ریشه‌ها به‌وسیله باکتری *P. fluorescens* و قارچ آنتاگونیست *P. lilacinum* در تیمارهای هر دو عامل و هر یک از عوامل بیوکنترل به‌تنهایی تفاوت معنی‌داری مشاهده نشد.

واژگان کلیدی: نماتد سیستی سیب‌زمینی، کنترل بیولوژیک، فرمولاسیون مایع، *Pseudomonas fluorescens* و *Purpureocillium lilacinum*

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