Plant growth promoting microbial consortia mediated classical biocontrol of sunflower necrosis virus disease

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ABSTRACT

Biological control is gaining momentum in the management of sunflower necrosis virus disease (SNVD) as no other effective method is available. In glasshouse experiment-I, six different plant growth promoting microbes (PGPM): *Streptomyces* sp. PM5, *Trichothecium roseum* MML005, *Bacillus licheniformis* MML2501, *Streptomyces fradiae* MML1042, *Pseudomonas aeruginosa* MML2212 and *Bacillus* sp. MML2551 and 2% *Morinda pubescens* fruit extract applied individually (seed + foliar applications) along with sunflower necrosis virus (SNV) were evaluated in sunflower. Among the treatments, *B. licheniformis* (*Bl*), *Bacillus* sp. (*Bsp*), *P. aeruginosa* (*Pa*), *S. fradiae* (*Sf*) effectively increased the plant growth and significantly increased the reduction of virus titre, it ranging from 32.5% to 52.5%. In experiment-II, the above four effective PGPM (*Bl*, *Bsp*, *Pa* and *Sf*) were developed as consortia in all possible combination in this study and were applied along with SNV against SNVD. All the consortial treatments significantly reduced SNVD in virus titre with disease reduction and concomitant increase in growth promotion when compared to control. In experiment-III, the best PGPM consortia (PGPMC) were applied as seed + soil inoculations along with SNV to study the induction of systemic resistance enzymes. The four culture consortium significantly reduced the SNVD symptoms and virus titer with a concomitant increase in plant growth promotion and ISR enzymes compared to control. In experiment-IV, based on biocontrol efficacy and ISR against SNVD from the experiments I to III, the two more dominant PGPMC treatments were selected and evaluated against SNVD under field conditions. From these results, *Bl* + *Bsp* + *Pa* + *Sf* effectively reduced the SNVD and improved the plant growth and yield parameters with additional seed yield with income and benefit cost ratio when compared to farmer's practice. In conclusion, PGPM (*Bl*, *Bsp*, *Pa* and *Sf*) was found to be very effective against SNVD under glasshouse and field conditions.

Key words: Biological control, PGPM(C), sunflower, sunflower necrosis virus disease, ISR.

INTRODUCTION

Sunflower necrosis virus disease (SNVD) was first observed in Karnataka state of India during the year 1997. Subsequently, it spread rapidly to other states with the disease incidence ranging from 2 - 90% (Jain *et al.,* 2000; Lavanya *et al.,* 2005; 2009). Sunflower cultivation has been seriously hampered by an SNVD caused by sunflower necrosis virus (SNV). SNV is a single stranded circular RNA virus with isometric virions; the sunflower ilarvirus was related to TSV on the basis of coat protein gene sequence (Prasad Rao *et al.,* 2000; Bhat *et al.,* 2002b). A disease similar in nature to SNVD has been reported in the Netherlands (Dijkstra, 1983) and Australia (Brunt *et al.,* 1996). Consequently, the area under cultivation has been reduced significantly to 1.33 M ha with the total production of 0.733 M tones in 2000 - 2001 as against 2.0 M ha with a total production of 2.0 M tones in 1998 - 1999 (Bhat *et al.,* 2002a; Jain *et al.,* 2003; Srinivasan *et al.,* 2009). This has resulted in substantial economic losses due to SNVD, thus forcing the farmers to switch over to alternate crops. As the prevention strategy to SNVD (Jain *et al.,* 2003) reported the Imidacloprid is very effective to control the viral transmission. However, there is no other effective and suitable method against SNVD available in recent years. In this scenario, therefore the biological control is an eco-friendly disease control approach and could be a supplement or an alternative to chemical control. Several researchers have focused in various aspects to manage the SNVD in India (Ramaiah *et al.,* 2001; Bhat *et al.,* 2002a-c; Jain *et al.,* 2003; Shirshikar, 2008; Srinivasan *et al.,* 2009).

Application of biocontrol agents (BCAs) or plant growth promoting rhizobacteria (PGPR) is considered as an important approach in crop protection against plant pathogens. Several microbes have been studied extensively as BCAs against various phytopathogens and these also showed plant growth promotion activity (Singh *et al.,* 2003; Lucy *et al.,* 2004; Mathivanan *et al.,* 2005; Srinivasan, 2007). In particular, the application of BCAs or PGPR strains greatly reduced the viral diseases in many crops (Murphy *et al.,* 2003; Martinez-Ochoa

et al., 2004; Kloepper *et al.,* 2004 a, b; Kandan *et al.,* 2005; Srinivasan *et al*., 2005; Srinivasan and Mathivanan 2009). Recently, the mixture of microorganisms with diverse activities against plant virus diseases is gaining momentum. PGPR strains were tested individually and in combinations (two/ more strains) for biological control against multiple plant pathogens (Raupach and Kloepper, 1998; Yan *et al.,* 2002; Idris *et al.,* 2007; Kavino *et al.,* 2007; Srinivasan and Mathivanan, 2009; Harish *et al.,* 2008, 2009).

Induction of systemic resistance (ISR) in plants was reported as one of the important mechanisms of viral disease suppression by BCAs or PGPR. PGPR have been evaluated under greenhouse and field conditions for ISR against several viral pathogens in various crops (Raupach *et al.,* 1996; Zehnder *et al.,* 2000; Murphy *et al.,* 2003). In contrast, the protective effects of the PGPR treatments described previously. Ton *et al*. (2002 a, b) reported that PGPR did not induce the resistance against turnip crinkle virus in *Arabidopsis*. These findings have emphasized that elaborate studies are required to establish the important role of ISR against a specific viral disease. The use of PGPR in the management of viral diseases under field condition is of very recent trend as reported from tomato mottle and spotted wilt viruses in tomato (Murphy *et al.,* 2000; Kandan *et al.,* 2005), cucumber mosaic virus in cucumber, tomato and pepper (Zehnder *et al.,* 2000; Murphy *et al.,* 2003; Kloepper *et al.,* 2004 a, b), banana bunchy top virus in banana (Kavino *et al.,* 2007; Harish *et al.,* 2008; 2009). Therefore, the present study aimed at investigating with SNVD control and plant growth promotion in various approaches such as (i) PGPM applied with seed + foliar applications against SNVD; (ii) the effective PGPM treatments developed with consortia and these consortia were evaluated against SNVD; (iii) to study the defense related enzymes induced by PGPMC treated plants and (iv) the effective consortial treatments tested against SNVD under field conditions.

MATERIALS AND METHODS

Maintenance of SNV

SNVD affected sunflower plants were collected from Dharmapuri and Erode districts of Tamil Nadu, India, which are the hotspot for this disease (Jain *et al.,* 2003). Preparation of virus inoculum and mechanical inoculation (Ramaiah *et al.,* 2001; Srinivasan, 2007) were carried out for the maintenance of SNV in sunflower plants in the present study.

Preparation of microbial strains

Six PGPM strains and plant fruit extract (*M. pubescens*) were obtained from the culture collection of the BioControl and Microbial Metabolites Laboratory, Centre for Advanced Studies in Botany, University of Madras, Chennai, India. The PGPM strains were cultured in 100 ml of their respective medium in 250 ml Erlenmeyer flasks at room temperature in a shaker at 150 rpm. The bacterial strains were grown for 48 hr and the actinomycetes and fungal strains were grown for 6 days. The PGPM cultures were harvested after their growth periods and used for the greenhouse experiments. An aqueous fruit extract of *M. pubescens* (2%) was also included as one of the treatments.

Experiment-I

Six different treatments (10^8 cfu/ml) and *M. pubescens* fruit extract were evaluated [seed treatment on 0 day, foliar application (15 days old seedlings) and the SNV (20 days old seedlings)] for SNVD control and plant growth promotion under greenhouse conditions. The treatment details are clearly mentioned in table 1.

Experiment-II

The four selected PGPM were harvested and prepared as liquid microbial consortia by mixing equal volume of each strain before use and evaluated against SNVD control.

Experiment-III

The five different PGPMC treatments were selected based on experiment-II. The PGPMC (seed and soil applications on 0 day were challenged with SNV inoculation on 15 day-old sunflower plants) were evaluated for plant growth promotion, SNVD control and the induction of systemic resistance enzymes.

Effect of PGPM on plant growth and SNVD

In seed treatment, sunflower seeds (Mordan variety, susceptible for SNVD) were surface sterilized with 1% sodium hypochlorite for 3 min, washed with distilled water and blot dried. The surface sterilized seeds were soaked separately in PGPM (experiment-I), PGPMC (experiment-II) and selected PGPMC (experiment-III) inoculums contained (10^8 cfu/mL) for 5 h. The treated seeds were air dried and sown in earthern pots at the rate of 10 seeds per pot. The seeds soaked in sterile water served as controls. Seed germination percentage was recorded with standard formula after 10 day-old of seed sowing. Foliar application, the PGPM and *M. pubescens* fruit extract (experiment-I) and PGPMC (experiment-II) was sprayed to sunflower plants at a rate of 50 ml per pot on 15 day-old sunflower plants (12.5 mL/plant) (Srinivasan *et al.,* 2009). In soil application, the selected PGPMC treatments (experiment-III) were applied in soil on 0 day, before the sowing the seed (Srinivasan, 2007). The SNV inoculation (pathogen) was administrated to 20 day-old sunflower plants (Ramaiah *et al.,* 2001).

Greenhouse experiments (I to III) were conducted in complete randomized block design (CRBD) with three replications (four plants/replication) to evaluate the biocontrol potential of PGPM and their consortia on plant growth promotion, ISR enzymes and SNVD control. The experiments were performed three times and totally 90 plants were used for seed germination study and 36 plants were tested for each treatment. Totally 72 plants (12 plants/treatment) were carried out for plant growth promotion, disease reduction, ELISA and ISR enzymes study. The plant growth parameters and disease severity were determined in all the treatments on 45 day-old sunflower plants (Copeland, 1998; Srinivasan and Mathivanan, 2009).

Field trial (Experiment-IV)

Multiplication of PGPM

The selected PGPM strains (*B. licheniformis* MML2501, *Bacillus* sp. MML2551, *P. aeruginosa* MML2212 and *S. fradiae* MML1042) were grown on 200 ml of their respective medium in 500 ml conical flask, the flasks were incubated on a rotary shaker at 150 rpm for 24 h for bacteria and 144 h for *S. fradiae* MML1042. The PGPM cultures were harvested and used for developing the liquid formulations. To each 1 litre of sterile solution containing 2.0% polyvinyl pyrollidine, 1.5% polyethylene glycol and 2.5% glycerol, 500 ml of the respective microbial culture was mixed separately under aseptic condition and stored in sterile plastic bottles at room temperature. Samples were taken to determine viable microbial population immediately after preparation of the liquid formulations and again at the time of application.

Preparation of PGPM consortia (PGPMC)

Equal amounts of liquid formulations of PGPM (10^9 cfu/ml) were mixed thoroughly for the preparation of PGPMC-1 and PGPMC-2, just before use. The PGPMC-1 contained *Bl + Bsp + Pa + Sf* and PGPMC-2 contained *Bl + Bsp + Pa*.

Liquid formulations of PGPMC against SNVD

Two different field experiments were conducted between the months of June and September (favorable season for SNVD) in the years 2005 and 2006 to study the efficacy of liquid formulations of the above PGPMC-1 and PGPMC-2 in farmers' fields at Dharmapuri district of Tamil Nadu, India. The individual plot size was 4 x 3 m^2 with a spacing of 45 cm between rows and 30 cm between plants. The experiments were conducted using randomized block design (RBD) with the following four treatments and with six replications of each treatment. T1: seed treatment + soil application + foliar spray of PGPMC-1; T2: seed treatment + soil application + foliar spray of PGPMC-2; T3: farmers' practice (seed treatment + foliar spray of imidacloprid and mancozeb); T4: control (water).

Applications of liquid formulations of PGPMC

In soil application, 3 ml of liquid formulation of PGPMCs were mixed with 240 g of farmyard manure (FYM) and applied to soil in a respective plot before sowing. There was no soil treatment of imidacloprid and mancozeb in farmers' practice. FYM with respective formulation materials excluding PGPM was applied in the control plots. In seed treatment, sunflower seeds were soaked with liquid formulations of PGPMCs (at 10 ml/kg of seeds) and shade dried for 5 hr. In farmers' practice, seeds were dressed with imidacloprid at 0.5 g/kg seeds and

Table 1. Effect of different PGPM and *M. pubescens* fruit extract on plant growth promotion and SNVD control in sunflower

Treatment	% seed germination	% increases of plant growth promotion			% increases of reduction of virus titre
		Shoot length	Root length	Vigour index	
Streptomyces sp. PM5	74.7	4.4^{e}	6.8^{g}	10.9^{f}	7.1^{h}
T. roseum MML003	70.7	0.3^{g}	16.5^{f}	3.9^{g}	21.6^{g}
B. licheniformis MML2501	82.7	37.8^{b}	123.3^{a}	83.4^{a}	38.7^{c}
S. fradiae MML1042	77.3	25.8^{d}	76.7^{bc}	50.0^{d}	32.5^{de}
P. aeruginosa MML2212	80.7	33.3^{c}	34.0^{bc}	52.4^{c}	43.2^{b}
Bacillus sp. MML2551	83.7	41.4^{a}	71.8^{bc}	75.4^{b}	52.5^{a}
M. pubescens	75.3	2.8^{f}	23.3^{d}	14.3^{e}	25.3^{f}
Water (control)	70.7	-	-	-	-

Values are mean of three replications. All the values were analyzed by one-way ANOVA.
Means in the columns of letter(s) in common are not significantly different at p = 0.05.
% seed germination = Number of seed germination/total number of seed sown X 100
% increases of plant growth parameters and reduction of virus titre = Treated - control/control X 100

mancozeb at 5 g/kg of seeds. The respective formulation materials devoid of PGPM treated seeds were used in controls. After the treatments, seeds were sown in their respective plots. Foliar spray, 3 ml of liquid formulations of PGPMCs were suspended separately in 300 ml of water and sprayed in a plot at different intervals on 15, 30 and 45 days old sunflower plants. Imidacloprid (0.05%) and mancozeb (2.0%) were sprayed in the farmers' practice and water with formulation materials excluding PGPM was sprayed in the control plots. All the recommended agronomical practices such as irrigation and weeding were conducted to raise the crop. Twenty five plants were selected at random and tagged in each plot for disease score. The SNVD scoring was carried out at 55 days using the following 0-9 scale (Srinivasan and Mathivanan, 2009). Seed germination was estimated after 10 days of sowing and the plant height was measured on 55 day-old plants. The crop was harvested at maturity and the yield parameters were determined.

Statistical analyses

The present experimental data were analyzed using analysis of variance (ANOVA) by Agres Statistical Software Package Version 3.01 (Agres, 1994). The least significant difference (LSD) analysis was performed to separate the group mean when ANOVAs were significant at $p = 0.05$.

RESULTS

SNV induced the typical symptoms such as scattered chlorotic lesions, mottle, vein clearing, severe necrosis, leaf distortion and necrotic streaks in inter-veinal areas and petioles after 15 days and the disease symptoms were prominent after 20 days. In experiment-I, among the treatment tested, *Bacillus* sp. increased highest seed germination rather than by *B. licheniformis* (Table 1). The treatment with *Bacillus* sp. increased the shoot length maximum up to 50.9 cm followed by *B. licheniformis*, *P. aeruginosa* and *S. fradiae*. In the case of root length, the maximum increase was recorded in *B. licheniformis* treated plants followed by *S. fradiae*, *Bacillus* sp. and *P. aeruginosa* in which the root length was recorded as 23.0, 18.2, 17.7 and 13.8 cm respectively. The sunflower seedling vigour index was consistently high in plants that received a treatment of PGPM and *M. pubescens* fruit extract compared to control. Maximum vigour index was estimated by the application of *B. licheniformis* followed by *Bacillus* sp. (Table 1). Results of ELISA indicated that SNV was present in all the plants that received PGPM and *M. pubescens* fruit extract treatments including control plants. However, a lower titre value was recorded in *Bacillus* sp. as against control plants. The virus titre values ranged from 0.721 to 1.18 in the rest of the treatments. The per cent decrease of virus titre ranged from 7.1 to 52.5 as compared to the water-treated control.

In experiment-II, the statistical analysis of the results represents that the PGPMC treatments significantly increased the different kinds of plant growth promotion and reduced the disease progress curve of SNVD more than in the case of the control plants. The applications of PGPMC significantly reduced the SNVD compared to control.

In experiment-III, seed + soil applications of PGPMC significantly promoted the percent increases of seed germination and plant growth promotion against control. The percent infection of SNVD affected plants showed high variation among the treatments. PGPMC were very effective and increased the disease reduction compared to control. In experiment III, seed + soil applications of the PGPMC along with pathogen (SNV) induced a greater amount of enzymes when compared to control.

In experiment-IV, application of PGPMC-1 improved maximum seed germination, head size, head weight, seed weight/head, 1000 seeds weight, seed yield/plot and total seed yield/ha compared to control (Table 2).

DISCUSSION

Control of plant virus diseases is a pressing need for the sustainable agriculture in the 21st century. Current practices for controlling plant viral diseases are mainly based on genetic engineering, plant breeding and chemical insecticides/ pesticides. There is a demand for developing new methods to supplement the existing disease management strategies to achieve better disease control in agriculture. The BCAs or PGPRs exercise various mechanisms to accomplish disease control; among them the induction of systemic resistance shows promising results against multiple plant diseases particularly against viral diseases (Benhamou *et al.,* 1998; Enebak and Carey, 2000; Hammerschmidt*et al.,* 2001; Lenardon *et al.,* 2005). Several researchers have reported that the PGPR strains provided better protection against virus diseases under greenhouse and field conditions (Zhender *et al.,* 2000; Kandan *et al.,* 2005). Therefore, we have investigated the potential of different PGPM for plant growth promotion and SNVD control. Among the treatments (PGPM and plant extract) evaluated, *Bacillus* sp., *B. licheniformis*, *P. aeruginosa* and *S. fradiae* effectively prevented the SNV infection. The ELISA data indicated the lower titre value for localized infection in the PGPM and plant extract treated plants than the control plants.

The ultimate goal of our study was to identify a combination of PGPM which would provide high level disease protection in sunflower against SNV. The PGPM could provide protection against plant pathogens when they are used individually. However, there might be variability in their performance due

Table 2. Effect of liquid formulation of PGPMC on seed germination, SNVD and yield parameters in sunflower

Treatment	% seed germin -ation	Disease score** (0 – 9 scale)	Head size (cm)	Head weight (g)	Seed weight/ head (g)	1000 seeds weight (g)	Seed yield/ plot (kg)	Total seed yield (kg/ ha)	Addi-tional seed yield over control (kg/ha)	Addi-tional income over control (Rs./ha)	Benefit of cost ratio
PGPMC-1 (*Bl* + *Bsp* + *Pa* + *Sf*)	24.4[a]	51.4[a]	55.2[a]	33.4[a]	64.1[a]	36.4[a]	64.0[a]	52.6[a]	936	12168	6.8
PGPMC-2 (*Bl* + *Bsp* + *Pa*)	21.4[b]	43.1[b]	47.3[b]	28.5[b]	50.0[b]	27.3[b]	40.0[b]	36.3[b]	628	8164	4.6
Farmer's practice (imidacloprid + mancozeb)	7.8[c]	30.6[c]	19.4[c]	10.5[c]	35.9[c]	15.2[c]	24.0[c]	16.5[c]	252	3276	3.7
Control (water)	-	-	-	-	-	-	-	-	-	-	-

Bl: *B. licheniformis* MML2501; *Bsp*: *Bacillus* sp. MML2551; *Pa*: *P. aeruginosa* MML2212; *Sf*: *S. fradiae* MML1042. cm: centi meter; g: gram; kg: kilo gram; ha: hectare.

All the values are mean of six replications and analyzed by one-way ANOVA using Agres Statistical Software Package Version 3.01 (Agres, 1994).

Values in a column followed by different letters are significantly different at p = 0.05.

*Values in brackets are % increase compared to control (% increase= treated – control/control).

**Values in brackets are % decrease compared to control (% decrease= control - treated/control). Rs.: Rupees; kg: kilo gram; ha: hectare. 12.5 kg seeds used for sowing/ha;

PGPMC-1 (*Bl* + *Bsp* + *Pa* + *Sf*) and PGPMC-2 (*Bl* + *Bsp* + *Pa*) applied as seed treatment at 10 ml/kg seed; soil application at 2.5 l/ ha; foliar applications at 2 l/ha/spray;

Microbial strain cost Rs. 150/kg. Farmer's practice (imidacloprid + mancozeb): seed treatment (seeds were dressed with imidacloprid at 0.5 g/kg of seeds +

mancozeb at 5 g/kg of seeds); foliar application, imidacloprid (0.05%) + mancozeb (2.0%) were sprayed in this treatment.

Control (water): Sterile water used in all the applications (seed treatment, soil and foliar applications).

Imidacloprid applied as seed treatment at 5 g/kg seed and foliar spray at 125 g/ha/spray; Imidacloprid cost Rs. 500 / kg;

Mancozeb applied as seed treatment at 5 g/kg of seeds and foliar spray at 625 g/ha/spray; Mancozeb cost Rs. 220 kg.

Labour: For seed treatment: 1 man h/ha; Soil application: 4 man h/ha; Foliar spray: 2 man day/ha/spray;

Labour cost at Rs. 65/day; Sprayer hire charge at Rs. 20/spray; Sunflower seed price is calculated at Rs. 13/kg.

to environmental conditions. In our study, we found that the individual treatments of PGPM resulted in different levels of SNVD control in sunflower. From the present investigation it is clear that *B. licheniformis*, *Bacillus* sp., *P. aeruginosa* and *S. fradiae* have performed well in the management of SNVD in sunflower. In this context, the information thus obtained from the present study would help in the successful development of a PGPM consortia for the effective SNVD management in sunflower.

The lowest disease score and virus titre were recorded in *Bl* + *Sf* + *Pa* + *Bsp* treated plants. Many reports indicated that combining fluorescent pseudomonads and *Bacillus* spp. with other BCAs resulted in effective control of plant diseases (Zehnder *et al.*, 2001; De Boer *et al.*, 2003; Mathivanan *et al.*, 2005). In disease control, the treatment (*Bl* + *Sf* + *Pa* + *Bsp*) was more effective in reducing the SNVD than control. SNV disease symptom was not detectable on young leaves after 45 days of PGPMC treated plants, in which there was no viral symptoms or mild necrosis symptoms. The absence of disease

symptoms in the newly formed leaves suggested that the disease resistance in sunflower was induced systemically by PGPMC treatments. In general, the absence of the disease symptoms clearly demonstrated that the microbial strains might be associated with plants that could induce one or more biochemical compounds, which act simultaneously in a synergistic manner to suppress the pathogen during infection.

On the whole, our analysis of the field experiments has indicated that the PGPMC-1 significantly increased the plant growth, yield and reduced the SNV disease incidence compared to PGPMC-2, farmers' practice and control. It was already established that the strains of *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp., improved plant growth in different crops (Chung*et al.,* 2005; Ryu*et al.,* 2005; Ji *et al.*, 2006) and recently *B. licheniformis* MML2501, *Bacillus* sp., *P. aeruginosa* and *S. fradiae* MML1042 have been reported to increase the plant growth parameters in sunflower (Srinivasan *et al.,* 2009), and in rice (Shanmugaiah *et al.,* 2006). The application of PGPMCs effectively increased the plant growth parameters and reduced the incidence of SNVD and eventually improved the yield attributes in field conditions. Maximum increase in seed yield with an additional income and benefit cost ratio was recorded in PGPMC-1 treatment. Hence, this formulation can be recommended to the farmers as one of the crop protection strategies for the management of SNVD and this practice may also be extended to other crops.

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