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Management of tomato spotted wilt virus (TSWV) and its thrips vector in tomato using a new commercial formulation of *Pseudomonas fluorescens strain* and neem oil

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Abstract
Tomato spotted wilt virus is spread by the insect vector *Thrips tabaci* and causes severe yield losses. Talc based powder formulation of *Pseudomonas fluorescens* strains with or without chitin, along with 3% Neem oil (Natural Pesticide) was applied to tomato plants. The present study deals with different methods of application of talc based powder formulations of *Pseudomonas fluorescens* strains to seed, soil and foliage or as a seedling dip followed by three foliar sprays of 3% Neem oil from seeds of *Azadirachta indica* (Bio pesticide), which reduced the number ofthrips vectorviz., nymphs and adults and also reduced the incidence of TSWVin tomato plants both in glasshouse and field conditions. We observed increased growth in PGPR tomato plants. DAC ELISA tests showed low concentration of viral antigen in PGPR treated tomato leaves and also in nymphs and adult thrips. An environmentally safe approach helped in managing the viral disease and optimizing tomato yield.

Keywords: TSWV, Thrips*tabaci*, *Pseudomonas*, 3% Neem oil, Chitin, DAC ELISA, Tomato

1. Introduction
Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable grown worldwide. The major constraint in tomato production is insect vector- transmitted virus diseases. General symptoms include necrotic spotting of leaves, stem necrosis, wilting, leaf curl/distortion, stunting, dieback of growing tips, poor fruit set, spots or rings on the fruit, irregular ripening, line patterns and mosaic of leaves, premature leaf drop. TSWV is transmitted by at least 11 species of thrips, including: *Frankliniella fusca*, *F. occidentalis*, *F. schultzei*, *Thrips palmi*, *T. setosus*, *T. tabaci.* The virus is also transmissible by mechanical inoculation, grafting, seed transmission in tomato. Contact insecticides are often not effective against thrips due to oviposition in tissue, the presence of adults inside tight buds, and migration of surrounding plants. Systemic insecticides often do not kill thrips fast enough to slow epidemics. Existence of insecticide resistant thrips biotypes and lack of resistant varieties of tomato to TSWV pose threat to farmers. Induced systemic resistance may be considered as the best option for management of insect-transmitted diseases. Fluorescent pseudomonads, non-pathogenic rhizobacteria, are among the most effective biological control agents against soil-borne plant pathogens (Kloepper and Schroth, 1978). *Pseudomonas* strains induced resistance not only against fungal and bacterial, but also viral diseases [6, 10, 14, 19, 25].
Neem Products are derived from Neem tree and act as powerful Insect Growth Regulators (IGR) and also help in controlling several nematodes and fungi. Neem products reduce insect growth in crops and plants [23]. Azadirachtin is the active ingredient neem oil and seed extracts which possess germicidal and anti-bacterial properties. They are useful to protect the plants from different types of pests. Neem based pesticides do not have residual effects [1]. Biological control gives better control of plant diseases by depending on less chemical pesticides and are more ecofriendly in nature.

2. Materials and methods
Isolation *of Pseudomonas fluorescens* strains from rhizosphere soils of different crops using King's medium B (KMB), followed by Physical tests like gelatin liquefaction, arginine dihydrolase, nitrate reduction and various carbon source utilization [12, 21, 22]. Two *Pseudomonas* isolates were identified and used in this experiment.

To maintain the culture KMB agar slants were prepared and the bacteria was streaked over it [7]. The bacterial strains were multiplied in KMB broth for 48 h and bacterial cells were collected by centrifugation at 8000*g* for 10 min and suspended in $MgSO_4$ and the population was maintained at 3×10^8 colony forming units (CFU) by using spectrophotometer [21].

2.1.1. Chitin amendments with talc-based formulations

Five grams of crab shell chitin (Sigma, USA) were slowly added to 100 ml of cold 0.25 N Hcl with vigorous stirring and kept overnight at 48C. The mixture was filtered through glass wool into 200 ml of ice cold ethanol at 48ºC with rapid stirring. Chitin suspension was centrifuged at 10,000 rpm for 20 min and by using distilled water, the chitin pellets were washed repeatedly, until we get neutral pH. The concentration should be adjusted to 10 mg/ml and added to KBB (1% v/v). Then the liquid medium with chitin should be autoclaved and the two *P. fluorescens* strains were grown in the medium for 48 hrs. 400 ml of liquid medium with chitin is added to 1 kg of Talc and mixed well and shade dried and later utilised for experiment [16, 19].

2.1.2. Preparation of Neem oil Solution

Neem oil extracted from mills from neem seeds were used in this experiment. 3 ml of Neem oil /litre of water and mixed with emulsifierand sprayed thrice, underneath the leaves, where thrips reside. Knapsack sprayers were used to spray neem oil solution in the field [1, 2, 3].

2.1.3. Insect Vector studies in Glasshouse - Maintenance of viral inoculum

Tomato seeds were sown in Mud pots filled with farmyard manure and 20, 30, 45 DAS plants were selected for virus inoculation through thrips vectors [13, 15]. The TSWV infected tomato plants were collected from the nearby farmer's field and inoculated into healthy tomato plants using thrips collected from infected plants and the pots were kept in insect proof rearing cages in a glasshouse and maintained throughout the experiment.

2.1.4. Mechanical Inoculation of TSWV by wedge grafting in Glass house

45 day tomato plants were inoculated with TSWV using infected scion through wedge grafting method. Disease grade scale from 1 to 5 was used for grading different symptoms expressed by TSWV. Percentage of TSWV infection was calculated and percent disease reduction over control was calculated [13, 20].

2.1.5. Thrips rearing cages.

Thrips vector is collected from chilli and Tomato fields by a fine small brush, for identifying thrips hand lens are required as they are present in lower surface of leaves and laidin tomato plants kept in rearing cages for 48 hrs. They are continuously maintained in cages and made viruliferous, so that they can infect PGPR treated tomato and control plants in a glasshouse. An inoculation period of 24 hrs is given to viruliferous thrips to inoculate the virus into PGPR treated plants. All the tomato plants were infected with TSWV by these thrips [15].

2.1.6. Seed Bacterization

Seeds of tomato (*Lycopersiconesculentum* Mill.) cultivar CO-3 were surface-sterilized with 1% sodium hypochlorite for 30 s and then rinsed in sterile distilled water and dried under a sterile air stream. Ten ml of bacterial inoculum containing 3×10^8 CFU/ml was added to Petri plates. To this, 100 mg of carboxyl methyl cellulose was added as adhesive material. Ten gram of seeds were soaked in 10 ml of bacterial suspension for 12 h. Then, the bacterial suspension was drained off and the seeds were dried overnight in sterile Petri plates [12, 21, 22].

2.1.7. Field studies

The talc-based formulations of single strain of *P. fluorescens* strains with or without chitin were also evaluated under field conditions. A field area at keerambur, Tamil Nadu, endemic with TSWV was selected and the trials were conducted in 2014 and 2015. The weather conditions were conducive for the rapid spread of TSWV in tomato by thrips. The trial was conducted as a randomized complete block design with three replicate plots treatment. The plot size selected was around 4x 3.5 m^2.Seed treatment, seedling root dip, soil application and foliar spray of *P. fluorescens* strains and also foliar spray of neem oil 3 % in specific treatments were done as described before. No pesticides were applied in PGPR treatments except Neem oil 3 % and the area was hand-weeded. The crop was irrigated every 6 days. Natural incidence of TSWV was recorded 20, 30, 45 and 60 days after planting. The effects of *P. fluorescens* on growth (plant height, plant biomass) and yield (fruiting cluster/plant, average fruit weight, number of fruits/plant and total yield) were also recorded. The data were analyzed statistically (Duncan Multiple Range Test).

2.1.8. Disease incidence and severity.

Disease incidence (percentage of infected plants) was assessed by examining the plants for TSWV symptoms. Plants were also scored individually for symptom severity using a 0-5 rating scale (0=No symptoms and 5=Severe symptoms) and severity for each plot were calculated. Records on disease incidence and severity level were taken at weekly or bi weekly intervals, starting one week after transplanting. Data on disease incidence were transformed to arcsine and analyzed using the analysis of variance procedure [13, 15].

2.1.9. DAC ELISA

A standard direct antibody coat (DAC) ELISA method was adopted to detect the viral antigen as described [8]. Microtitre plates (Tarsons Pvt. Company, India) were coated with the capture antibody, a TSWV specific polyclonal antiserum (Germany) was diluted with 0.05 M sodium carbonate buffer (pH 9.6) to a dilution of 1/1000, with 100 mL added well as described [8]. Absorbance was determined at 405 nm using a Bio-Rad model 3550 (Microplate reader, USA).

3. Results

The results revealed an effective management of thrips nymphs and adults, which transmitted TSWV in Tomato. Talc-based formulations of PGPR strains with chitin were applied to seed, seedling and soil, foliar followed by three foliar sprays with Neem oil 3% (instead of spraying PGPR strains in T_3 and T_2) at 10 days interval was considered as the best treatment (Pf1+ Chitin + 3% Neem oil) showed very low disease incidence and percent disease reduction over control was recorded as 68.85, under glasshouse conditions (Table 1). Percent reduction in number of thrips over control were recorded as 70.45 in T_3 (Table 1), under glasshouse conditions. Moreover, the number of plants infected was less

in PGPR treated tomato plants compared to untreated control (Table 1). In the current research T_3 (Pf1++ Chitin + 3% Neem oil) showed very low TSWV disease incidence (12.5, 19.9, 16.7 in three field trials) and low number of insect vectors compared to other PGPR treatments, insecticide and untreated control. In all trials, the Pf1+ Chitin + 3% Neem oil strains (59.63) with chitin showed lower symptoms of TSWV and high fruit yield (1.80 kg/Plant) compared to other treatments. (Table 2) and the thrips population were very low (7 and 9.5) even under field conditions in T3 and T_2 treatments respectively. (Table 3)

In this current research 3% Neem oil as a foliar spray in PGPR treated tomato plants added advantage. It effectively managed both thrips nymphs and adult population (See Table 3) which in turn, reduced TSWV incidence and increased fruit yield both under glasshouse and field conditions. Especially with Pf1+ chitin + 3% Neem oil combinations, performed better. In the present study, the DAC-ELISA test was performed for the detection of TSWV in PGPR treated tomato plants and the ELISA values were found to be lower in PGPR treated leaf samples especially in Pf1 + + Chitin + 3% Neem oil compared to the untreated control plants. Apart from these, T SWV was detected in thrips nymphs and adults collected from PGPR treated tomato plants and untreated control. (Fig. 1).

4. Discussions

Single strains of *Pesudomonas* with chitin showed better control of TSWV and its thrips vector in tomato. This finding is in line with previous research work of several researchers [9, 10, 14] and delayed TSWV symptom expression up to ten additional days from the day of challenge inoculation were observed under greenhouse conditions [18, 19, 20]. Moreover, the number of plants infected was less in PGPR treated tomato plants compared to untreated control. These results agree with previous studies of three scientists in the management of tobacco necrosis virus (TNV) in tobacco, cucumber mosaic virus (CMV) in tomato, tomato mottle virus (ToMoV) in tomato and tomato leaf curl virus (iTLCV) in tomato, respectively [9, 10, 14]. PGPR-mediated ISR has been exploited for the management of virus diseases successfully under field conditions [19, 25]. Earlier reports revealed reduced ToMoV incidence and disease severity in PGPR treatments [10]. Commercial development of PGPR+chitosan was formulated and evaluated for plants to manage the disease [16, 25]. Management of TSWV uses different PGPR strains, i.e. individual strains in tomato under field conditions was reported by two researchers [6, 20]. All the above research data for the management of virus diseases supported our findings in the management of TSWV with PGPR strains. The efficacy of PGPR strains significantly reduced the TSWV symptoms in tomato under field conditions.

The results in this study confirmed the research work done by several authors, who had reported for reduction in population densities of whitefly and aphid on cabbage [2]. They noted that aqueous leaves and fruit extracts from *Melia azedarach* L. (Meliaceae), a close relative of neem, produced greater mortality of whiteflies than on control plants [3]. Similarly, leaf extracts of neem previously resulted into significant mortality of bean aphids [2, 3, 17]. Active ingredient found in neem tree is Azadirachtin, which act as an insect repellent and insect feeding inhibitor, thereby protecting crop plants. This ingredient belongs to an organic molecule class called tetranortriterpenoids. This compound is similar to insect hormones (Ecdysones) and also control the process of metamorphosis when the insects pass from larva to pupa to adult stage [4, 24].

Thrips obtained from PGPR treatment (Pf1 + chitin+ 3% Neem oil) showed slightly lower values, when tested for ELISA, compared to untreated control. Nymphs recorded more virus titre than the adults. This has been reported earlier in these research works [18, 19, 20]. She tested the PGPR treated tomato against TSWV and iTLCV infected samples randomly through indirect ELISA and DAS ELISA and reported for less disease symptoms and low virus titre in PGPR treatments than untreated control under field conditions. Further several researchers reported for successful management both virus and vector through ISR [9, 10, 14, 25]. In addition to suppression of the disease, the PGPR treatment greatly induced the plant growth. Both under greenhouse and field experiments, the PGPR treated plants showed increased yield of the tomato plants compared to the untreated control plants (Tables 1 and 2). Similar results were reported in two experiments [11, 22]. Thus, in our present study, we observed better action of PGPR strains with chitin along with 3% Neem oil in managing TSWV in the tomato and its vector population in an ecofriendly way.

5. Conclusion

Plant virus diseases transmitted by insect vectors can be managed effectively through the combined effects of Induced systemic resistance and a natural bio pesticide. ISR by *Pseudomonas* was effective against virus infections and bio pesticide checked the insect population and thereby recorded a reduced disease incidence. For controlling both the virus and insect- vector this combination of ISR and Neem oil, would be a best alternative in future.

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Table 1: Effect of PGPR strains, with Neem oil and insecticide on TSWV and insecticide on the Occurrence of disease incidence percentage in glasshouse studies.

S. no	Treatments	Percentage disease incidence	Percent reduction over control	No of Thrips	Percent reduction over control
T_1	Pf1+ TSWV	30.7[d]	56.14	10.0[d]	54.45
T_2	Pf1+ Chitin + TSWV	26.6[e]	62.00	8.0[e]	63.63
T_3	Pf1+ Chitin + Neem oil 3% + TSWV	21.8[f]	68.85	6.5[f]	70.45
T_4	Neem oil 3% alone	40.1[b]	42.71	13.5 [b]	40.90
T_5	Dimethoate (0.04%) + TSWV	49.0 [c]	30.00	13.0 [b]	40.99
T_5	Dimethoate (0.04%) + Neem oil 3% + TSWV	44.0 [b]	37.14	12.0 [c]	45.45
T_6	Control (untreated) + TSWV	70.0[a]	0.00	22.0[a]	
T_7	Healthy control	0.00	-	0.00	-

Each value is the mean of three replicates. Percentage data were arcsine transformed prior to ANOVA. Means followed by same letter do not differ significantly at the 5% probability level by DMRT. Numbers in the parenthesis are arcsine transformed values.

Table 2: Effect of PGPR treatment with Neem oil and insecticide on TSWV in tomato under field conditions.

S. No	Treatments	Trial I	Yield kg/plant	Trial II	Yield kg/plant	Trial III	Yield kg/plant	Average disease incidence	Percent reduction over control
T_1	Pf1+ TSWV	20.4 ^{bc}	1.50^{e}	28.5^{d}	1.48^{c}	26.4^{d}	1.56^{e}	25.1^{cd}	38.27
T_2	Pf1+ Chitin + TSWV	17.6^{cd}	1.60^{d}	24.5^{e}	1.64^{f}	23.3^{d}	1.58^{e}	21.8^{d}	46.21
T_3	Pf1+ Chitin + Neem oil 3% + TSWV	12.5 ^{e}	1.90^{f}	19.9^{f}	1.80^{g}	16.7^{e}	1.83^{f}	16.36^{e}	59.63
T_4	Neem oil 3% alone	25.3 ^{b}	1.45^{c}	34.2^{c}	1.30^{cd}	32.8 ^{bc}	1.40^{d}	30.76^{b}	24.01
T_5	Dimethoate (0.04%) + TSWV	25.8 ^{b}	1.12^{b}	34.8^{c}	1.27^{c}	31.5^{b}	1.28 ^{c}	31.30^{b}	22.77
T_5	Dimethoate (0.04%) + Neem oil 3% + TSWV	23.5 ^{b}	1.40^{c}	31.2^{b}	1.35^{d}	29.2 ^{b}	1.37^{d}	27.96^{c}	31.01
T_6	Control (untreated) + TSWV	35.8 ^{a}	0.85 ^{a}	44.3^{a}	0.80^{a}	41.5^{a}	0.77^{a}	40.53^{a}	-
T_7	Healthy control	0.00	1.14^{b}	0.00	1.13^{b}	0.00	1.15^{b}	-	-

Individual and strain mixtures were applied as seed treatment, root dipping, soil application and foliar spray. The data were arcsine transformed before analysis. Data followed by the same letter in a column are not significantly different by DMRT at the 5% level.

Table 3: Effect of PGPR treatment with Neem oil and insecticide, on TSWV insect vector (thrips) in tomato under field conditions.

S.no	Treatments	No. of Adult Thrips	Percent reduction in no of Thrips over control
T_1	Pf1+ TSWV	11.0 ^{c}	59.25
T_2	Pf1+ Chitin + TSWV	9.5^{d}	64.81
T_3	Pf1+ Chitin + Neem oil 3% + TSWV	7.0^{e}	74.40
T_4	Neem oil 3% alone	15.0 ^{b}	44.44
T_5	Dimethoate (0.04%) + TSWV	15.5 ^{b}	42.59
T_6	Dimethoate (0.04%) + Neem oil 3% + TSWV	14.5^{b}	46.29
T_7	Control (untreated) + TSWV	27.0 ^{a}	-
T_8	Healthy control	-	-

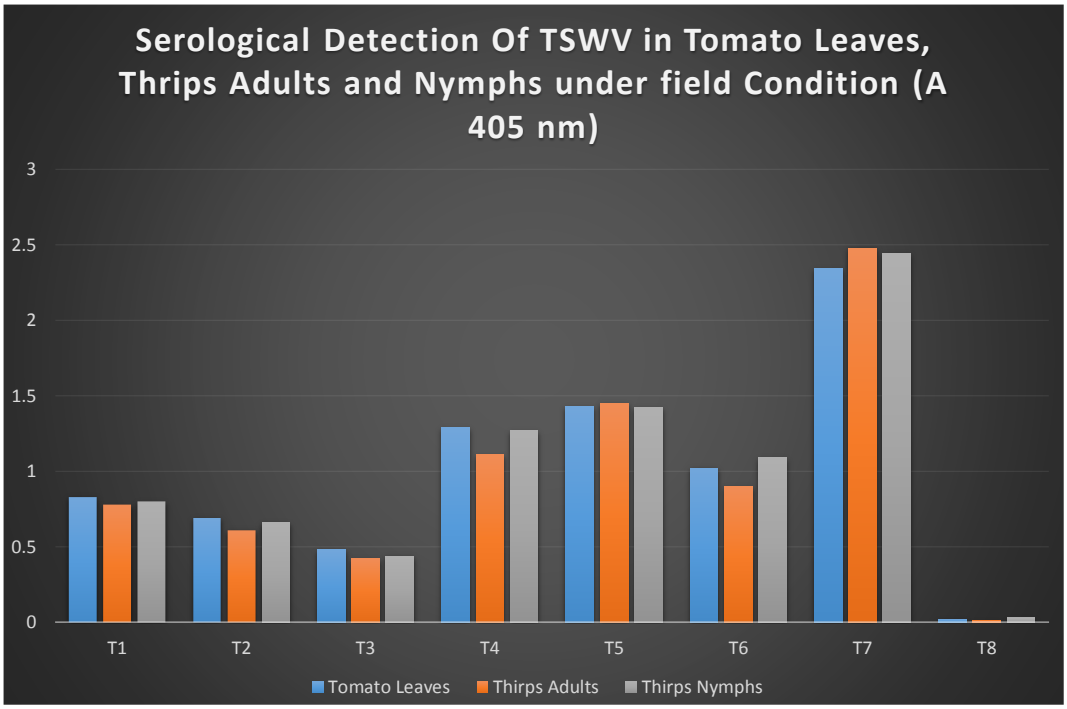

Fig 1: Serological detection of TSWV in PGPR + chitin strains, Neem oil and insecticide treatments in tomato, thrips adults and nymphs under field conditions. (A 405 nm).Values are the mean of three replicates. Error bar indicate mean + SE. Treatments are: (1) Pf1+ Chitin + TSWV. (2) Pf1+ Chitin + Neem oil 3% + TSWV (3) Pf1+ Chitin + Neem oil3% + TSWV (5) Dimethoate (0.04%) + TSWV (6) Dimethoate (0.04%) + Neem oil 3% + TSWV (7) Control (untreated) + TSWV (9) Healthy control.

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