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Full Length Research Paper

Pseudomonas fluorescens as an efficient entomopathogen against *Oligonychus coffeae* Nietner (Acari: Tetranychidae) infesting tea

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The biocontrol efficacy of the bacterium, *Pseudomonas fluorescens* was evaluated against the red spider mite (RSM), *Oligonychus coffeae* in the laboratory. Both bacterial suspension and extra cellular filtrate of 24, 48 and 72 h old were sprayed to test the efficacy of bacterium against adult mites. The mortality due to *P. fluorescens* increased as the age and percentage of culture increased. Chitinase activity of *P. fluorescens* in response to chitin induction was measured in nutrient agar by estimating the amount of reducing sugars liberated. Chitinase activity was higher in 72 h old culture.

Key words: *Pseudomonas fluorescens*, red spider mite (RSM), tea, biocontrol, chitinase.

INTRODUCTION

The red spider mite (RSM), infesting commercially cultivated tea plants, *Camellia sinensis* L. (O. Kuntze), is a typical colonizing species characterized by very high rate of population increase (Muraleedharan et al., 2005). This mite normally infests the upper surface of mature tea leaves and in severe infestation, they move even to the lower surface of mature leaves as well as to tender tea shoots. As a result of feeding, the maintenance foliage turns ruddy bronze, rendering infested fields distinct even from a distance. Severe infestation ultimately leads to defoliation (Selvasundaram and Muraleedharan, 2003). As the continuous use of synthetic acaricides for the management of red spider mite may result in environmental pollution, possible development of pesticide resistance in the target pest, pesticide residues in made tea and also the devastation of natural enemies, eco friendly control methods deserve attention.

Alternate control strategies have been suggested for the management of *Oligonychus coffeae* in South India (Selvasundaram and Muraleedharan, 2003; Babu, 2009). Biological control agents like insect parasitoids, predators and fungal pathogens play a vital role in the natural regulation of many tea pests. The minor status of many tea pests in India is obviously due to the influence of these biological control agents (Muraleedharan and Selvasundaram, 1995).

Pseudomonas aeriginosa (Poinar and Poinar, 1998) and *Pseudomonas putida* (Aksoy et al., 2008) have been reported to cause disease in spider mite, *Tetranychus urticae.* Bacterial chitinases have been reported to be effective in controlling the insects and mites by hydrolyzing chitinous exoskeleton (Kramer and Muthukrishnan, 1997). Their role as defensive agents against organisms such as insects, nematodes and fungi has evoked in-terest in researchers on the studies related to chitinolytic enzymes (Sahai and Manocha 1993). Chitin, (1, 4 – β – linked polymer of N – acetyl – β – D – glucosamine (GlcNAc)) is reported to be the second most abundant polymer in nature after cellulose. It forms the major component of exoskeleton and gut linings of insects; hence chitin metabolism can be an excellent target for selective pest management strategy (Kramer et al., 1997). Chitinolytic enzymes and their genes have gained

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Table 1. Effect of 24 h old *P. fluorescens* culture on red spider mite adults.

Treatment	% Mortality after			
	24 h	48 h	72 h	96 h
50% culture	22±3.74^{b}	40±5.48^{b}	66±8.12^{b}	76±7.48^{b}
50% extra cellular	42±4.90^{c}	62±7.35^{c}	72±8.00^{b}	86±7.44^{b}
75% culture	66±9.27^{d}	86±9.27^{d}	90±7.75^{c}	94±6.00^{cd}
75% extra cellular	72±5.83^{d}	92±3.74^{d}	100±0.00^{c}	100±0.00^{d}
100% culture	100±0.00^{e}	100±0.00^{d}	100±0.00^{c}	100±0.00^{d}
100% extra cellular	100±0.00^{e}	100±0.00^{d}	100±0.00^{c}	100±0.00^{d}
Control	0±0.00^{a}	0±0.00^{a}	0±0.00^{a}	0±0.00^{a}

Means followed by the same letter do not differ significantly at $P = 0.05$ according to DMRT.

attention in the recent years because of the importance of chitin and its metabolic enzymes in insect growth and development. Their chemical, physical, kinetic and regulatory properties, as well as their potential for utilizing them as microbial agents are being explored (Kramer et al., 1997).

The objective of the present study was to evaluate the efficacy of the saprophytic soil bacterium *Pseudomonas fluorescens*, as a bio control agent against RSM infesting tea, while its chitinase activity is also examined.

MATERIALS AND METHODS

The organism

A *P. fluorescens* strain, previously isolated from tea soil collected from UPASI tea experimental farm in a biodegradation study of pesticide dicofol (Sarkar et al., 2009) with the gene bank accession number GQ900589 was used in the present study as a biocontrol agent.

Stock culture of red spider mite

Adults of *O. coffeae* were collected from the tea fields of UPASI tea experimental farm. Leaves with spider mites were immediately transferred onto one-year old potted tea plants grown under green-house conditions and used as stock culture. From the stock, adults were transferred onto fresh tea leaf squares (6 × 6 cm) placed on moistened cotton pads (0.5 inch thick) in plastic trays (42 × 30 × 6.5 cm). Rearing trays were kept under controlled conditions of 25±1°C, 75±5% RH and 16 L: 8 D photoperiod. Withered and dry leaves were regularly replaced.

Bioassay

The effect of *P. fluorescens* was evaluated against adults of red spider mite adopting two different methods: i) spraying of bacterial suspension and ii) spraying of extra cellular filtrate of the bacterium using different aged cultures (24, 48 and 72 h) at the concentrations of 50, 75 and 100%. The experimental arena consisted of 2 cm diameter tea leaf discs placed on moist cotton in Petri plates (9 cm diameter). Each treatment was replicated five times with ten mites per replication. In the first method, the mites were introduced on the leaf discs and the suspension was sprayed from a distance of 25 to 30 cm with a hand spray atomizer of 50 ml capacity until the leaf surface got just wet with very fine droplets. For the second method (extra cellular application), the bacterial culture was centrifuged at 10000 rpm for 10 min at 4°C and the crude supernatant was col-lected and sprayed as described above. Leaf discs treated with sterile media served as control. Dead mites were counted at 24 h interval till 120 h.

To analyze the factor contributing to the mortality of mites by extra cellular filtrate of *P. fluorescence,* chitin assay was carried out in the laboratory by screening chitin clearing zone in plates. For this study, freshly grown bacterial strain as well as a strip of filter paper dipped in extra cellular bacterial filtrate were patched onto plates of chitin agar medium (Monreal and Reese, 1969) amended with chitin (Sigma – Aldrich Chemicals) and incubated for 5 to 7 days at 27±2°C. The plates were observed for chitin clearing zones around the patched bacterial colonies. The zones were then visualized by flooding chitin agar plates with Congo red solution (0.2% aqueous) for 15 min. The plates were then drained and again flooded with 1 N NaCl for 15 min (Teather and Wood, 1982).

Enzyme assay

A loop-full of *P. fluorescens* (10^{-9}) was taken and transferred to 100 ml nutrient broth and incubated for 24 h in a shaker (150 rpm) at 37°C. Then it was centrifuged at 10000 g for 15 min. The super-natant was collected and used as the enzyme source. The activity of chitinase was determined by the quantitative estimation of the reducing sugars produced with the colloidal chitin as appropriate substrate of enzyme assay (Sun et al., 2006). The reaction mixture was prepared with 0.5 ml enzyme solution and 0.5 ml of 1% colloidal chitin in 1 ml McIlvaine buffer (100 mM/L citric acid, 200 mM/L sodium phosphate) at a pH of 5.8. The mixture was incubated for 30 min at 36°C using a shaking water bath and the reaction was stopped in boiling water for 10 min. The amount of reducing sugars released in the supernatant was measured by the method using dinitrosalicylic (DNS) acid reagent, and the absorbance was measured at 540 nm using spectrophotometer (LABINDIA® UV 3000^{+}). One unit (U) of chitinase activity was defined as the amount of enzymes that liberated 1 µmol of the reducing sugar per minute. N- acetyl glucosamine (GlcNAc) served as standard.

RESULTS

Bio assay

The results of present study indicated the biocontrol efficacy of *P. fluorescens* against RSM (Tables 1 to 3).

Table 2. Effect of 48 h old *P. fluorescens* culture on red spider mite adults.

Treatments	% Mortality after			
	24 h	48 h	72h	96 h
50% culture	56±6.78^{b}	72±7.35^{b}	86±5.10^{b}	88±5.83^{b}
50% extra cellular	72±3.74^{b}	82±4.90^{b}	90±5.48^{b}	96±4.00^{bc}
75% culture	82±5.83^{ca}	96±4.00^{c}	100±0.00^{c}	100±0.00^{c}
75% extra cellular	90±4.47^{ae}	100±0.00^{c}	100±0.00^{c}	100±0.00^{c}
100% culture	100±0.00^{e}	100±0.00^{c}	100±0.00^{c}	100±0.00^{c}
100% extra cellular	100±0.00^{e}	100±0.00^{c}	100±0.00^{c}	100±0.00^{c}
Control	0±0.00^{a}	0±0.00^{a}	0±0.00^{a}	0±0.00^{a}

Means followed by the same letter do not differ significantly at $P = 0.05$ according to DMRT.

Table 3. Effect of 72 h old *P. fluorescens* culture on red spider mite adults.

Treatment	% Mortality after			
	24 h	48 h	72 h	96 h
50% culture	64±2.45^{b}	74±4.00^{b}	90±3.16^{b}	94±4.00^{b}
50% extra cellular	74±2.45^{c}	86±2.45^{c}	94±2.45^{b}	100±0.00^{c}
75% culture	84±4.00^{a}	96±2.45^{a}	100±0.00^{c}	100±0.00^{c}
75% extra cellular	92±3.75^{e}	100±0.00^{a}	100±0.00^{c}	100±0.00^{c}
100% culture	100±0.00^{e}	100±0.00^{a}	100±0.00^{c}	100±0.00^{c}
100% extra cellular	100±0.00^{e}	100±0.00^{a}	100±0.00^{c}	100±0.00^{c}
Control	0±0.00^{a}	0±0.00^{a}	0±0.00^{a}	0±0.00^{a}

Means followed by the same letter do not differ significantly at $P = 0.05$ according to DMRT.

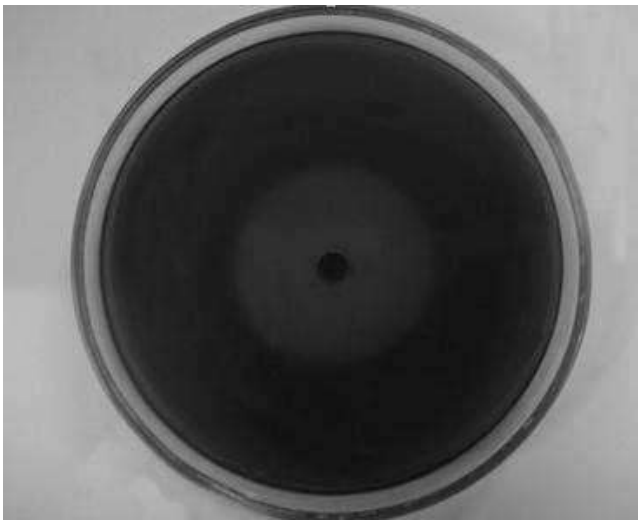

Figure 1. Chitin clearing zone of *P. fluorescens* – extra cellular effect.

Red spider mites showed reduced mobility and cessation of feeding after the application of *P. fluorescens* and the body fluid began oozing out. 100% culture achieved cent percent mortality in 24 h with one day old culture, where-as 75% culture yielded 100% mortality in 72 h when 48 h old culture was used. 50% culture was found to be least efficient even when 72 h old culture was used. In accordance to Koch's postulates, the strain was reisolated from the dead insects to confirm its efficacy as a suitable biocontrol agent.

The mortality due to extra cellular filtrate could be due to the chitinase enzyme present in the cell free filtrate which was confirmed by the positive result of chitin clearing zone study. The chitin around the strip of filter paper dipped in extra cellular filtrate was cleared by the chitinase enzyme in the filtrate (Figure 1).

Enzyme assay

The filtrate produced in the presence of colloidal chitin showed chitinase activity whereas the control filtrate did not show any chitinase activity up to 24 h. Production of chitinase was found to be higher in 72 h old culture (Figure 2).

DISCUSSION

The results of this study revealed the potential of *P. fluorescens* as a microbial biocontrol agent by causing significant mortality of *O. coffeae*. The significance of

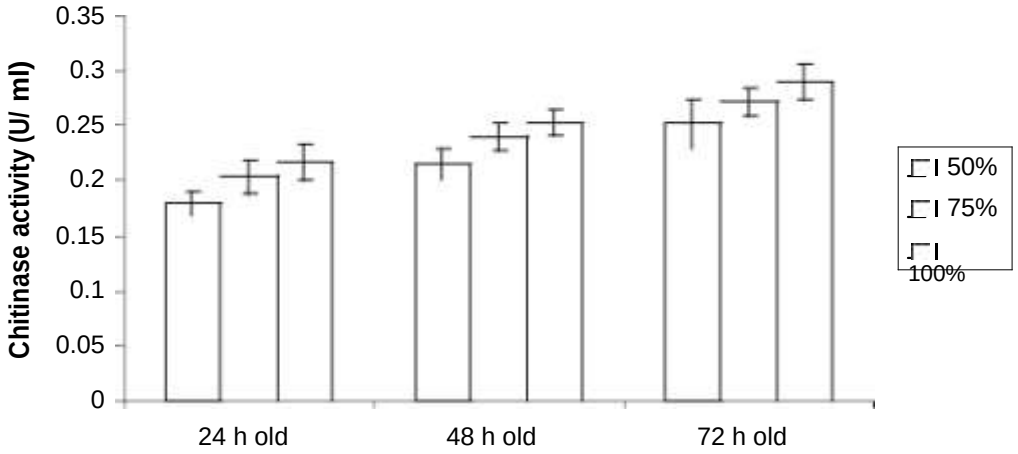

Figure 2. Chitinase activity of *P. fluorescens* in different hours.

different cell surfaces virulence factors as responsible component for the effective colonization by the patho-genic bacteria has been highlighted by Vodovar et al. (2006). The cells of *P. fluorescens* were introduced on to the body surface of mites either indirectly by brushing their ventral surface against the surface of leaf disc or by cleaning their mouthparts. As the enzyme contents are more predictable and controllable and less energy is required for the production, microorganisms are preferred to plants and animals as sources of enzymes (Pandey et al., 1999). The easy availability of raw materials with constant composition for their cultivation also makes fungi a reliable source for chitinase.

In the present study, the utilization or degradation of colloidal chitin by the isolate indicated its chitinolytic activ-ity and ability to utilize the degraded chitin monomers and oligomers as a substrate for growth and multiplication. It is possible that the colloidal nature of the medium might have offered ease to the bacterial culture to metabolize it. Several strains of *Trichoderma harzianum* and *P. fluorescens* were earlier screened for chitinase produc-tion based on chitinolytic activity on agar medium containing colloidal chitin as carbon source (Viswanathan et al., 2003). The entry of entomopathogens in to the tissues of insect pests, which are susceptible, is supported by the chitinases that cause perforations in the membranes (Brandt et al., 1978). The formation of lytic zones in chitin amended medium is because of the presence of lytic enzyme, chitinase in the cell free culture filtrate. *Pseudomonas entomophilia* exhibited virulence against *Drosophila melanogaster* due to strong hemolytic activity, involving enzymes such as lipases, chitinases and or hydrolases (Vodovar et al., 2006). Research on bacteria mediated insect control has revealed that bacterial chitinases may hydrolyze the insect's chitin (Broadway et al., 1998). In the present study, many of these factors might have contributed to the mortality of red spider mites caused by *P. fluorescens*. Further studies on formulation, shelf life and field bioefficacy of this bacterium have to be conducted for utilizing it as a successful biological control agent against red spider mite.

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