INFLUENCE OF CHITIN ON POPULATION DYNAMICS AND CHITINASE ACTIVITY OF ANTAGONISTIC BACTERIA

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Abstract: The effect of chitin on population and chitinase activity of antagonistic bacteria were studied under *in vitro* condition. *P. fluorescens* (BIB_2) and *B. subtilis* (BIL_8) strains in the presence of chitin in liquid medium increased the population of bacterial antagonists. The highest level of bacterial population (200.33×10^8 cfu $ml^{-1}$of broth) was observed in chitin containing medium inoculated with *P. fluorescens* (BIB_2) strain. Whereas, chitin containing medium inoculated with *B. subtilis* (BIL_8) strain recorded the bacterial population of 141.33×10^8 cfu $ml^{-1}$of broth. Chitin amendment significantly increased the chitinase activity of both the antagonistic bacterial strains. Of these, *P. fluorescens* (BIB_2) responded well to the addition of chitin and produced highest amount of chitinase (7.39 nmol of GlcNAc min^{-1} ml^{-1}) in chitin amended medium. Whereas, *B. subtilis* (BIL_8) strain produced 3.92 nmol of GlcNAc min^{-1} ml^{-1} of chitinase in chitin amended medium. *P. fluorescens* (BIB_2) + chitin was found to be superior in supporting the survival of *P. fluorescens* and recorded a population load of 17.00×10^7 cfu $g^{-1}$of product at 150 days after storage in talc based formulations. Further the population of *P. fluorescens* (BIB_2) slightly increased from 41.00×10^7 cfu $g^{-1}$of product to 45.33×10^7 cfu $g^{-1}$of bioformulation at 100 days after storage.

***Keywords*: Chitin, Antagonistic bacteria, Population and Chitinase activity.**

Introduction

Normally fungicides are the primary means of controlling plant diseases. But the fungicides are under special scrutiny for posing potential oncogenic risks (Eckert and Ogawa, 1985). The increased consumer preference for healthy agricultural products and environmental risks associated with chemical residues in food are the major driving forces for the search of new safer control methods. Over the past few decades, biological control has emerged as an effective strategy to combat the decay of fruits. Plant growth promoting rhizobacteria (PGPR) especially *Pseudomonas fluorescens* (Ardakani *et al.,* 2010) and *Bacillus subtilis* are promising candidates as bioprotectants (Ramamoorthy *et al.,* 2001; Mahadtanapuk *et al.,* 2007). Though remarkable success has been achieved in this direction through the use of antagonistic microorganisms, the information generated on the performance of the introduced antagonists into the ecosystem under varying field conditions still remains inadequate constituting a major obstacle in the large scale adoption of this technology.

Recently more emphasis has been laid on supplementing various nutrients with bioprotectants, which is better than either alone (Janisiewicz or Bors, 1995). It implies several good attributes by enhancing the antagonist's multiplication, survival rate for effective establishment in the field and subsequent fruit rot control. Recently a potential approach in biocontrol involves the use of the natural bioactive substances which inhibits fungal growth and also activates the biological efficiency of the antagonistic microorganisms. Chitin is a naturally occurring high molecular weight linear homo polysaccharide composed of N-acetyl-D glucosamine residues in α (1-4) linkage. Chitin and its derivatives are biodegradable and biocompatible natural polymers with a wide range of uses in cosmetology, food industry, biotechnology, medicine and agriculture (Li *et al.,* 1997). Chitin can be found in a variety of species in both the animal and plant kingdoms. The traditional source of chitin is shellfish waste from shrimp, antarctic krill, crab and lobster processing (Thirunavukkarasu *et al.,* 2011). Involvement of chitin adjuvant in improving the efficacy of various antagonists and triggering the plant originated ISR either alone or in combination with biocontrol agents has been demonstrated in various crops (Vivekananthan *et al.,* 2004; Viswanathan and Samiyappan, 2008; Loganathan *et al.,* 2010). With this background, this study was formulated to assess the effect of chitin on population and chitinase activity of antagonistic bacteria.

Materials and methods

Isolation of bacterial antagonists

Antagonistic bacteria were isolated from leaf surface, fruit skin and blossom of mango collected from major mango growing areas of Tamil Nadu using leaf washing technique (Gould *et al.,* 1996). A small plant material was mixed with 5 ml of sterile distilled water in a flask which was shaken on a shaker for 30 min. Then 1 ml of suspension was added to a Petri plate containing nutrient agar medium and incubated at room temperature (28 ± 2°C) for 48 h. The growing colony was subcultured on nutrient agar (NA) using single colony isolation. The slant was kept at 10°C in refrigerator and used as stock culture. Totally 52 isolates of bacteria were isolated and among them 30 were isolated from leaf surface, 6 were isolated from blossom and 16 were isolated from fruit skin and designated as BIL (Bacterial Isolate from Leaf), BIB (Bacterial Isolate from Blossom) and BIF (Bacterial Isolate from Fruit), respectively. Based on the observations on colony morphology and biochemical tests, only 22 isolates showed positive results. Out of this 22 bacterial isolates the biochemical tests confirmed, 12 isolates were identified as

Pseudomonas fluorescens and 10 isolates were identified as *Bacillus subtilis*. Based on the dual culture technique and poison food technique *P. fluorescens* (BIB_2) and *B. subtilis* (BIL_8) strains were selected for the further studies.

***In vitro* effect of chitin on bacterial antagonists**

Preparation of colloidal chitin

Five grams of crab shell chitin was slowly added into 100 ml of cold 0.25 N HCl with vigorous stirring and kept overnight at 4°C. The mixture was filtered through glass wool into 200 ml of ice cold ethanol at 4°C with continuous stirring. The resultant chitin suspension was centrifuged at 1000 rpm for 20 min. and the chitin pellets were washed repeatedly with distilled water until the pH become neutral. The conc. of colloidal chitin was adjusted to 10 mg ml^{-1}.

Testing the effect of chitin on bacterial population

One loopful of 48 h. old *P. fluorescens* (BIB_2) and *B. subtilis* (BIL_8) cultures were inoculated into King's B broth and Nutrient broth amended with 1% chitin, respectively. Three replications were maintained for each treatment and a suitable control was maintained without any amendment for comparison. After the inoculation, conical flacks were incubated in a mechanical shaker under constant shaking at 150 rpm for 48 h. at room temperature (28 ± 2°C). After the incubation period the bacterial population ($\times 10^8$ cfu ml^{-1} of broth) was calculated following the serial dilution plating technique.

Effect of chitin on chitinase activity

Antagonistic bacteria grown in chitin amended broth was used for this study. Chitinase activity in bacterial culture was assayed calorimetrically according to the procedure by Boller and Mauch (1998). The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg ml^{-1}). After incubation for 2 h. at 37°C, the reaction was stopped by centrifugation at 1000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (w/v) snail gut enzyme (Helicase) for one h. After one h., the reaction mixture was brought to pH 8.9 by the addition of 70 µl 1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min. and then rapidly cooled in an ice bath. After addition of 2 ml of dimethylamniobenzaldehyde (DMAB), the mixture was again incubated for 20 min. at 37°C. Immediately thereafter, the absorbance was measured at 585 ηm. N-acetylglucosamine was used as a standard. The enzyme activity was expressed as ηmol of GlcNAc equivalents $min.^{-1}$ ml^{-1} of bacterial culture.

Preparation of commercial formulation of biocontrol agents

A loopful of effective bacterial isolate was inoculated into the sterile King's B broth for *P. fluorescens* (BIB_2), Nutrient agar for *B. subtilis* (BIL_8) and incubated in a rotary shaker at 150 rpm for 72 h. at room temperature (28 ± 2°C). After 72 h. 400 ml of bacterial suspension containing 9×10^8 cfu ml^{-1}, one kg of the carrier material (talc powder), 15 g calcium carbonate and 10 g CMC were thoroughly mixed, shade dried to reduce the moisture content below twenty per cent and packed in polythene bags (Nandakumar *et al.*, 2001). For testing the combination effect of the talc based formulation of *P. fluorescens* (BIB_2) and *B. subtilis* (BIL_8) the individual formulations with the adequate cfu were mixed thoroughly at 1:1 (w/w) ratio just before application and sprayed.

Chitin amended talc based formulations of bacterial antagonists

Incorporation of colloidal chitin into broth medium and formulation development

Colloidal chitin was prepared as described earlier and incorporated into broth medium (1%, v/v) and the mixture was autoclaved at 15 psi for 30 min. Then the cultures were inoculated individually into their respective broth and kept in a shaker for 72 h. at room temperature (28 ± 2°C). After 72 h. of incubation, the broth containing 9×10^8 cfu ml^{-1} was used for the preparation of talc based formulation. To the 400 ml of bacterial suspension, 1 kg of the purified talc powder (sterilized at 105°C for 12 h.), calcium carbonate 15 g (to adjust the pH to neutral) and carboxy methyl cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions. The product was shade dried to reduce the moisture content (less than 20%) and then packed in polypropylene bags and sealed. At the time of application, the population of biocontrol strains in talc formulation was found to be 2.5 to 3×10^8 cfu g^{-1}. Chitin at 1% alone amended media without any inoculation of antagonistic bacteria was mixed with talc powder and used for the chitin alone treatment.

Population dynamics of bacterial antagonists in talc based formulation

A sample of one g of the product was drawn from each bioformulation just before packing as well as on 0, 25, 50, 75, 100, 125 and 150 days of storage for preparing the serial dilutions and the population of *P. fluorescens* (BIB_2) and *B. subtilis* (BIL_8) was estimated by serial dilution technique using Kings B and Nutrient agar medium respectively.

Results and discussion

Effect of chitin amendment on population of effective antagonistic bacteria

The results presented in table 1 showed that *P. fluorescens* (BIB_2) and *B. subtilis* (BIL_8) strains in the presence of chitin in liquid medium increased the population of bacterial antagonists. The highest level of bacterial population (200.33×10^8 cfu $ml^{-1}$of broth) was observed in chitin containing medium inoculated with *P. fluorescens* (BIB_2) strain. Whereas, chitin containing medium inoculated with *B. subtilis* (BIL_8) strain recorded the bacterial population of 141.33×10^8 cfu $ml^{-1}$of broth. The bacteria grown in the medium containing chitin + water and water alone recorded lowest bacterial population. In general, *Pseudomonas* and *Bacillus* are known to produce chitinase in culture medium and their production is further increased when the medium is supplemented with chitin source. Several earlier reports indicate the beneficial effect of chitin. The addition of inducers (chitin alone) increased the antagonist's population of *Pseudomonas* spp. in the culture medium (Rajkumar *et al.*, 2008). The replacement of glycerol in King's medium with colloidal chitin enhanced the bacterial population in addition to having enhanced antifungal activity against *C. falcatum* (Viswanathan and Samiyappan, 2001). The addition of chitin to soil leads to an increase in the population of chitinolytic microbes which in turn reduced the plant diseases caused by fungal pathogens (Benhamou *et al.*, 1996; Lafontaine and Benhamou, 1996). Manjula and Podile (2001) reported that a steady increase in cell numbers of

introduced *B. subtilis* was observed in chitin supplemented formulations. A population increase of 25% was observed in chitin containing medium inoculated with FP7 strain (Nandakumar *et al.,* 2007). The above results lend support to the present findings.

Chitinase activity of effective antagonistic bacterial strains grown in chitin amended medium

Chitinase enzyme produced by bacterial strains was assayed in culture filtrates of *P. fluorescens* (BIB_2) and *B. subtilis* (BIL_8) strains. Chitin amendment significantly increased the chitinase activity of both the antagonistic bacterial strains. Of these, *P. fluorescens* (BIB_2) responded well to the addition of chitin and produced highest amount of chitinase (7.39 nmol of GlcNAc min^{-1} ml^{-1}) in chitin amended medium. Whereas, *B. subtilis* (BIL_8) strain produced 3.92 nmol of GlcNAc min^{-1} ml^{-1} of chitinase in chitin amended medium (Table 1).

The increased population and the addition of chitin might have resulted in enhanced chitinase activity. Induction and enhanced activity of chitinase in the chitin containing medium have been reported previously (Ordentlich *et al.,* 1988; Frandberg and Schuurer, 1998; Viswanathan and Samiyappan, 2001). Watanabe *et al.* (1990) reported that six isoforms and 38 kDa of chitinases are produced by *B. circulans* grown in YNB (Yeast Nitrogen Base) medium containing 0.2% chitin. Among them, two isoforms had the highest colloidal chitin degrading activities. In *in vitro* assays, *B. subtilis* and *B. licheniformis* in combination with chitin showed antagonistic against *P. capsici* and *R. solani* and produced high levels of chitinase (Ahmed *et al.,* 2003). Addition of chitin induced one isoform in each strain (Nandakumar *et al.,* 2007) of *C. capsici.*

Population dynamics of antagonistic bacteria in talc based bioformulations

Antagonistic bacterial population was assessed in talc based formulations at different days of storage. *P. fluorescens* (BIB_2) + chitin was found to be superior in supporting the survival of *P. fluorescens* and recorded a population load of 17.00 $\times 10^7$ cfu $g^{-1}$of product at 150 days after storage. Further the population of *P. fluorescens* (BIB_2) slightly increased from 41.00 $\times 10^7$ cfu $g^{-1}$of product to 45.33 $\times 10^7$ cfu $g^{-1}$of bioformulation at 100 days after storage. Thereafter, the population declined steadily. This was followed by *B. subtilis* (BIL_8) + chitin which recorded 12.00 $\times 10^7$ cfu g^{-1} of bioformulation (Table 2).

A safe storage period of three months for maintaining the viability of *P. fluorescens* in talc was observed by Kloepper and Schroth (1981). Vidhyasekaran and Muthamilan (1995) found that *P. fluorescens* was viable for eight months in talc based preparation. The talc based formulation of *P. chlororaphis* and *B. subtilis* had a shelf life of more than four months (Kavitha *et al.,* 2005). Bora *et al.* (2004) reported that the population load of *P. putida* at the end of six months in talc based chitin amended formulation was 10^8 cfu g^{-1} of product. Similarly, Nakkeeran *et al.* (2006) reported that peat and talc based formulations of *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 survived for more than five months.

Shelf life of *S. griseus* in talc based formulation was stable up to 105 days (Anitha and Rabeeth, 2009). The chitin has been used to study improved activity of *P. fluorescens* on the assumption that the bacterial number and chitinolytic activity (capable of attacking the chitinaceous hyphal wall of pathogen) will be increased (Radja Commare *et al.,* 2010). All the above reports were in line with the present observations.

Table 1. Effect of chitin amendment on population and chitinase activity of antagonistic bacteria

T. No.	Treatments	Bacterial population (x 10^8 cfu/ml of broth)		Chitinase activity (nmol of GlcNAc min^{-1} ml^{-1})	
		P. fluorescens (BIB_2)	*B. subtilis* (BIL_8)	*P. fluorescens* (BIB_2)	*B. subtilis* (BIL_8)
T_1	Medium	161.00 ^{b}	111.00 ^{b}	5.72 ^{b}	3.36 ^{b}
T_2	Medium + chitin	200.33 ^{a}	141.33 ^{a}	7.39 ^{a}	3.92 ^{a}
T_3	Water alone	37.67 ^{c}	28.00 ^{d}	1.13 ^{d}	0.80 ^{d}
T_4	Water + chitin	44.33 ^{c}	36.00 ^{c}	2.40 ^{c}	1.11 ^{c}

Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT)

Table 2. Population dynamics of antagonistic bacteria in talc based bioformulations

T. No.	Treatments	Bacterial population ($\times 10^7$ cfu g^{-1})						
		0 DAS	25 DAS	50 DAS	75 DAS	100 DAS	125 DAS	150 DAS
T_1	*P. fluorescens*	27.00 ^{b}	29.00 ^{c}	31.66 ^{c}	35.33 ^{b}	38.00 ^{c}	16.66 ^{c}	7.33 ^{c}
T_2	*B. subtilis*	26.33 ^{c}	28.00 ^{d}	30.66 ^{c}	34.33 ^{b}	36.00 ^{d}	15.00 ^{d}	6.00 ^{d}
T_3	*P. fluorescens* + chitin	28.66 ^{a}	33.00 ^{a}	37.33 ^{a}	41.00 ^{a}	45.33 ^{a}	22.66 ^{a}	17.00 ^{a}
T_4	*B. subtilis* + chitin	28.00 ^{a}	31.33 ^{b}	35.00 ^{b}	39.60 ^{a}	41.33 ^{b}	20.00 ^{b}	12.00 ^{b}

DAS- Days after storage

Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT)

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