Research Paper :

Preparation and Shelf-life Study of *Pseudomonas* and *Bacillus* Bioformulations Against Phytopathogenic *Pythium* and *Fusarium* species

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SUMMARY

Four *Pseudomonas* and three *Bacillus* isolates from rhizosphere of healthy tomato and cotton plants with potent antifungal activity against phytopathogenic *Fusarium* and *Pythium* species were used to prepare bioformulations. Talc and dried fecal pellets (DFP) of sheep and goats were used as carrier materials, along with carboxymethylcellulose (CMC) as a sticking agent. The formulations were stored at room temperature (30^{0}C) and cold conditions (4^{0}C) and tested for viability, after each month. The bioformulations of *Bacillus* species were relatively more durable than *Pseudomonas* species. Storage of bioformulations at 4^{0}C was found significant than at room temperature. The DFP based formulations of *Pseudomonas* as well as *Bacillus* species were more durable than their talc based formulations. DFP was found to be the best carrier material for formulation of bacterial biocontrol agents.

Key words : Biocontrol formulation, *Pseudomonas*, *Bacillus*, Shelf-life, Phytopathogenic fungi

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India is an agro-based country and more than half of the total population either directly or indirectly depends on agriculture. The Indian farmers have to suffer great economic losses per year due to weather irregularities and crop diseases caused by phytopathogens such as pests, herbs, insects and microorganisms including-bacteria, fungi, algae, viruses etc. The soil-borne fungal pathogens of crops are most risky and cause substantial economic losses (Agrios, 2005). Control of crop diseases using chemicals available in the market with different trade names has become a regular practice since last half century. However, indiscriminate use of chemical agents to control the plant diseases since last few years has created great harm to human beings, animals, vegetation and the complete environment. Hence, a relatively safe and eco-friendly mean of disease control has become necessary. Biological control is the best solution.

The biocontrol products are prepared in the form of powder, pellets or wet formulations, applicable for seed coating, soil amendment and foliar spray. Preparation of biocontrol formulations involves primary screening of antagonists from disease suppressive soils against phytopathogens, by *in vitro* tests. Highly potent species of antagonists are selected in secondary screening and used to produce large biomass. A suitable carrier material such as dried fecal pellets (DFP) of sheep and goats, farmyard manure, gram shell and other agricultural wastes are used to prepare bioformulations using the biomass (Gaur *et al*., 2005; Bohra and Mathur 2005). The objective of the present work was to isolate *Pseudomonas* and *Bacillus* species with potent antagonistic activity against phytopathogenic fungi, particularly *Pythium* and *Fusarium* species, prepare biocontrol formulations, study their shelf-life and biocontrol efficiency.

MATERIALS AND METHODS

Isolation of phytopathogenic fungi and antifungal bacterial species:

According to the Koch's postulate-'the pathogen must be isolated and brought into pure culture and studied for its specific characters' (Rangaswami and Mahadevan, 2005). phytopathogenic *Pythium* and *Fusarium* species were isolated from infected tomato and cotton plants by tissue segment method (Agrios, 2005). *Bacillus* and *Pseudomonas* species were isolated from rhizosphere of healthy tomato and cotton plants using Nutrient agar (NA) and Kings B (KB) agar, respectively. Antifungal cultures were selected among the isolates by dual culture/ co-culture method (Krishnamurthy and Gnanamanickam 1998; Saikia *et al*., 2004). Potent antifungal isolates were identified on the basis of microscopic, cultural and biochemical characters as well as

16S r-RNA sequencing.

Preparation of bacterial formulations:

Cell mass- 100µl of each *Pseudomonas* and *Bacillus* culture was separately inoculated in 100ml NB in 250ml Erlenmeyer flasks and incubated at 28^{0}C for 48hrs. The broth cultures of four *Pseudomonas* species and three *Bacillus* species were mixed separately in equal amounts.

Carrier material- Two types of carrier materials were used for preparations of bioformulations *i.e.* Talc and dried fecal pellets (DFP) of goats and sheep. Carboxymethylcellulose (CMC) was used as sticking agent.

Types of bacterial formulations prepared:

Bf-1: Talc + CMC + Broth cultures of four selected *Pseudomonas* species.

Bf-2: Talc + CMC + Broth cultures of three selected *Bacillus* species.

Bf-3: DFP + CMC + Broth cultures of four selected *Pseudomonas* species.

Bf-4: DFP + CMC + Broth cultures of three selected *Bacillus* species.

Talc was adjusted at pH 7.0 using calcium carbonate. Dried fecal pellets (DFP) of sheep and goats were collected from pen and scrutinized. One kg of DFP and talc were separately filled in polyethylene bags and sterilized in autoclave at 121^{0}C for 30min. 10g sterile CMC was added in 100ml broth cultures of *Pseudomonas* and *Bacillus* species and mixed with talc and DFP in a sterile trays, dried approximately to 30%, and packed in polyethylene bags, in laminar air flow (Rangeshwaran and Prasad, 2000; Ramesh and Korikanthimath, 2004).

Study of shelf-life of bacterial formulations:

The bacterial formulations were stored at 4^{0}C and room temperature. Viability and population density of these formulations was tested after each month, up to one year. The bacterial formulations were suspended in sterile distilled water @ 1g/100ml (1:100). Two further dilutions *i.e.* 1:1000 and 1:10,000 were prepared in sterile distilled water. 100µl of each dilution was, respectively inoculated on NA plates by spread plate technique. The plates were incubated at 28^{0}C for 24hrs. Number of colonies appeared on NA for each dilution were counted and population density/g was calculated. Results are recorded in Table 1 and 2.

Population density/g = (Average number of colonies per plate) x (Dilution factor) x (10).

Table 1 : Viability tests for bacterial formulations stored at 4^{0}C

Test period (Months)	Population density of Bf-1 (CFU/g)	Population density of Bf-2 (CFU/g)	Population density of Bf-2 (CFU/g)	Population density of Bf-3 (CFU/g)
Zero	1.00 x 10^6	1.00 x 10^6	1.00 x 10^6	1.00 x 10^6
1	1.40 x 10^5	1.60 x 10^5	1.60 x 10^6	1.85 x 10^6
2	1.15 x 10^5	1.20 x 10^5	1.50 x 10^5	1.25 x 10^6
3	3.82 x 10^4	1.76 x 10^4	1.22 x 10^5	1.92 x 10^5
4	2.60 x 10^4	1.20 x 10^4	2.60 x 10^4	1.36 x 10^5
5	6.20 x 10^3	1.56 x 10^3	2.82 x 10^3	1.76 x 10^4
6	1.82 x 10^2	1.90 x 10^2	1.26 x 10^2	2.60 x 10^3
7	00	1.60 x 10^2	00	1.82 x 10^3
8	–	1.20 x 10^2	–	1.60 x 10^3
9	–	1.10 x 10^2	–	1.50 x 10^3
10	–	1.0 0 x 10^2	–	1.34 x 10^3
11	–	00	–	1.20 x 10^3
12	–	–	–	1.1 x 10^3

Values are average of triplicates tests.

Table 2 : Viability tests for bacterial formulations stored at room temperature

Test period (Months)	Population density of Bf-1 (CFU/g)	Population density of Bf-2 (CFU/g)	Population density of Bf-2 (CFU/g)	Population density of Bf-3 (CFU/g)
Zero	1.00 x 10^6	1.00 x 10^6	1.00 x 10^6	1.00 x 10^6
1	5.20 x 10^4	8.20 x 10^5	2.15 x 10^6	2.40 x 10^6
2	00	1.60 x 10^4	1.50 x 10^5	2.25 x 10^6
3	–	1.20 x 10^4	–	1.52 x 10^5
4	–	00	–	1.36 x 10^5
5	–	–	–	00

Values are average of triplicates tests

RESULTS AND DISCUSSION

The result obtained from the present investigation are summarized below :

Identification of phytopathogenic fungal isolates:

Isolate F1-White and fluffy mycelial growth on PDA was observed. Branched, hyaline, coenocytic mycelium, vesicles with zoospores, free flagellated zoospores were observed in stained preparations, under microscope.

Isolate F2- Light brown mycelial growth on PDA was observed. Branched, septate, hyaline mycelium and free sickle shaped, septate, hyaline macroconidia were observed in stained preparations, under microscope.

The growth pattern of phytopathogenic fungi on PDA and the microscopic characters of mycelium and spores putatively identified the fungal isolates as F1-*Pythium* species and F2- *Fusarium* species (Mukadam *et al*., 2006; Rani and Kumar, 2007). This indicated that, the

Fig. 1 :Primary Screening of Antifungal *Pseudomonas* Isolates

Fig. 1 : Secondary Screening of Antifungal Pseudomonas Isolates

Pythium and *Fusarium* species commonly cause infections to crops such as root rot, collar rot, damping-off, blights, fruit decay, wilts, etc. (Pathak *et al*., 2006). Among the soil borne fungal pathogens, *Fusarium* species are considered to be very dynamic and notorious pathogens (Naik, 2003). The *Pythium* species are relatively fast growing and aggressive fungal pathogens, particularly at moist and low temperature conditions (Loganathan *et al*., 2004).

Screening and identification of potent antifungal isolates:

Among total 300 bacterial isolates, eighteen *Pseudomonas* and twelve *Bacillus* isolates showed antifungal activity, of which four *Pseudomonas* and three *Bacillus* isolates were potent. These isolates were identified on the basis of microscopic, cultural and biochemical characters and 16S r-RNA sequencing as- *Pseudomonas aeruginosa*13, *P. aeruginosa* 58, *P. putida*71, *P. fluorescens*106 and *Bacillus thuringiensis* 184, *B. cereus* 220 and *B. subtilis*252.

Shelf-life of Pseudomonas and Bacillus formulations:

The initial population density of all these four bacterial formulations was 10^6/g. The shelf-life of two *Bacillus* formulations (Bf-2 and Bf-4) was 10 and 12 months, respectively, whereas it was 6 months for both the *Pseudomonas* formulations (Bf-1 and Bf-3), at 4^0C. However, at room temperature the values were found to be decreased to three and four months for *Bacillus* formulations and one and two months for *Pseudomonas* formulations Bf-2 and Bf-4, respectively. The shelf-life of *Pseudomonas* as well as *Bacillus* formulations was relatively prolonged in cold storage (4^0C) than at room temperature. At temperature below 5^0C, the metabolic activities of microorganisms are ceased and the cell division is almost stopped (Madigan and Martinko, 2006). Singh *et al*. (2000) observed that, shelf-life of the bioformulations of antagonists was affected by storage conditions. However, it is highly economic for the dealers and customers to use freeze or any other cold storage devices.

The cell count of the talc based *Pseudomonas* and *Bacillus* formulations (Bf-1 and Bf-2) stored at 4^0C indicated that, the initial population (10^6/g) decreased gradually up to 6 and 10 months, respectively (Table 1). In case of DFP based *Pseudomonas* and *Bacillus* formulations (Bf-3 and Bf-4) the colony forming units (CFU) per gram was increased during first month and then decreased subsequently for the next. The shelf-life of Bf-3 and Bf-4 was greater than that of Bf-1 and Bf-2, respectively. This indicated that, the DFP preparations were more durable than talc based. Some *Pseudomonas* and *Bacillus* isolates may able to grow at 4^0C. The bacterial growth may also be possible in this case due to increase in temperature of freeze during the 'cut-off' period of electricity.

The carrier material DFP is eco-friendly to add in soil and also economic. DFP may provide nutrients and enhance the growth of biocontrol bacteria and to some extent the plants also, when applied in soil. The shelf-life of these bacterial formulations varied with the carrier material used. The fecal pellets of goats and sheep, farm yard manure and agricultural wastes like gram shell are the best carrier materials.

The shelf-life for *Bacillus* formulations found more as compared to the shelf-life for *Pseudomonas*. This is certainly due to the spore forming ability of *Bacillus* species. Mandhare and Suryawanshi (2003) observed that, an antifungal air-borne antagonist *Bacillus thermophilus*

survive under ordinary conditions for more than 20 months.

Many microbial bioformulations are available in market with different forms such as liquid for spray, powder for seed coating and granules for soil application. Talc based formulations of *Pseudomonas* species were used by Krishnamurthy and Gnanamanickam (1998); Viveknathan *et al*., (2004); Ramesh and Korikanthimath, (2004); Singh and Sinha (2007). *Trichoderma* formulations were prepared and studied for biocontrol of crop plants with different carrier materials as $MgSO_4$ + CMC (Das and Hazarika, (2000), fecal pellets of goat and ships (Gaur *et al*., 2005), talc + CMC (Rangeswaran and Prasad, 2000; Vivekanathan *et al*., 2004; Khan and Sinha, 2005).

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