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Optimization of nutritional conditions and effect of time and temperature for the production of bacterial biocontrol agents

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Abstract

Yield potential is drastically affected by a number of soil borne fungal pathogens which result into quality and quantity reduction. Plant growth promoting rhizobacteria (PGPR) colonizing the rhizosphere result into disease control and also enhance the plant growth characters. The population dynamics of the bio-agents in prepared formulations viz., *Pseudomonas fluorescens* on vermicompost (PFV), *Pseudomonas fluorescens* on Organic matter (PFOM), *Bacillus subtilis* on Vermi compost (BSV) and *Bacillus subtilis* on Organic matter (BSOM) was determined at 7, 30, 60, 90 and 120 days after storage (DAS) at 5 °C and 28 °C. An increasing trend in colony forming unit was seen with the increase in time interval and maximum cfu (312.67 cfu) was recorded at 60 DAS in the treatments stored at 28 °C compared with (193.67 cfu) at 5 °C. Decrease in population was observed at 90 DAS and lowest bacterial population was recorded at 120 DAS at both 5 °C and 28 °C.

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Introduction

The pathogens indispensably persist in seed and soil and can survive in soil for six years (Nga *et al.*, 2010). Due to prolonged survival of the pathogenic fungi, only cultural control measures are not feasible and chemical control is costly due to repeated applications and also dangerous to the environment and humans. Up till now, no resistant cultivar is available in Barani regions. In Pakistan, about 10-50% losses by Fungal Root Disease have been reported on chickpea in the dry areas during the past several years (Woltz and Jones, 1981). Repeated applications of same fungicides results into residual effects and also not safe to human health thus results into acute and chronic toxicity (Goldman, 2008).

Keeping in view the health hazardous and toxic effects of synthetic chemicals, there is strong need to search out some alternate methods to minimize this loss which must be inexpensive and non-toxic to plants and with greater efficiency to controls the disease. Use of biological agents is most economical, and durable control measure to cope with the disease causing pathogens. Bio-control involves suppression of disease causing agents thus indirectly improve plant health (Lugtenberg and Kamilova, 2009).

A number of bio-control agents with effective bio-control activities were reported by a number of researchers which include fungal and bacterial agents. Synthetic chemicals have various limitations as these are health hazard and also result into environmental contamination (Ndoumbe-Nkeng and Sache, 2003). Biological control of plant pathogens is potentially preferable to chemical means.

The antagonistic bacteria, based on their genetic potential and physiological adaptation, tolerance drought, increasing salt concentration, and temperature fluctuations thus improve plant growth (Maheshwari *et al.*, 2012).

Utilization of antagonistic microorganisms as bio-control agents in agriculture is of increasing trend as an alternative to chemical pesticides. Utilization of microbial inoculants for the disease control and plant growth improvement is generally termed as bio-pesticide, as bacterial inoculants increase plant growth, enhance the nutritional contents of the host plant (Vessey, 2003) moreover, significant crop yield enhancement have been reported by the application of PGPR (Garcia de Salamone, 2000).

A number of bacterial and fungal agent based bio-pesticide formulation are commercially available for the effective control of diseases. Several registered products are: Biobit-WP, D-Stop (WP), Foray 48-B (WP), Novodor- FC (SC) and Wormax-of (WP). Viruses (Zucchini Yellow Mosaik Virus) and fungi (*Ampelomyces quisqualis*, *Candida oleophila*, *Trichoderma atroviride*, *T. asperellum*, etc.) are used in crop protection programs (Sanco, 2014).

Materials and methods

Preparation of rhizobacterial cell suspension

For the preparation of rhizobacterial cell suspension, 48 hours old cultures of rhizobacterial isolates on NA media were taken and the cultures were harvested in 10 ml distilled water separately. Cell suspensions of the cultures were subjected to serial dilution and were diluted from 10^{-1} to 10^{-5} in 9 ml distilled water. The cell concentration in the final dilution was adjusted to 10^{8}cfu/ml with the help of Spectronic-20 spectrophotometer.

Substrates collection for the preparation of rhizobacteria based bio-pesticide

For the mass multiplication of the rhizobacterial agents as bio-pesticide, Rice bran (Rb), wheat bran (W) and decomposed mustard oil cake (D) Farmyard manure (F), vermicompost (V) and rice straw (R) were purchased from the local markets of Rawalpindi, Pakistan.

Preparation of substrates

The collected substrates were firstly air dried for 14 days followed by proper grinding. Substrates were filled into polypropylene bags and 10 ml of sterile distilled water was also added. 3 packets of 100 g for each substrate were prepared.

Mass multiplication of rhizobacteria on different organic substrates

Prepared substrates packets were autoclaved and Carboxymethyl cellulose (1% aq) (C), polyvinyl alcohol (P) and white flour gum (W) were uses as adhesives for comparative study. Adhesive compound was added in substrate containing polypropylene bags and pH of the substrate was maintained 7 by using $CaCO_3$. Polythene bags were sealed with heat and sterilize @ 121 °C for 30 minutes. The prepared mixture was spread in sterilized disposable plates. Mannitol (8.5 ml of 3% mannitol for 100 g formulation) was added as osmoticant to maintain the osmotic pressure of the prepared substrate.

Prepared rhizobacterial (R) cell suspensions of concentration of 10^8 cfu/ml was pipetted into the substrate mixture (1:10 v/w) and thoroughly mixed by using sterilized spoon.

The bio-agent carrying mixtures were properly labeled with abbreviations (i.e., initial letter of each of the substrate carriers and adhesives as mention earlier along with the biocontrol agent (R) example CVR, PFR etc.). Each disposable plate containing bio-formulation was covered with another sterilize plate and incubated under optimal conditions. Prepared formulations were allowed to dry for 3 days at room temperature (Kloepper and Schroth, 1981). Each prepared formulation was divided into three parts, packed in polythene bags, heat sealed and stored at room temperature. In order to carry out shelf life studies, another set of prepared bio-formulations were store at 4 °C.

Influence of storage temperature and time on the shelf life of the rhizobacteria in different carrier materials

The population dynamics of the bio-agents in formulation forms was determined at different days after storage in the two storage conditions and population of rhizobacteria was measured by Dilution plate technique after given days of incubation period. Population of rhizobacteria in prepared formulations was determined at 7, 15, 30, 60, 90 and 120 days after storage (DAS) at room temperature.

For this, formulations were firstly stored at room temperature for 15 days to enhance the initial population of bio-agents and population was determined at 15 DAS at room temperature and later at 30, 60, 90 and 120 DAS at 4 °C.

Population dynamics were observed by mixing 1 g of aseptically in formulations with 10 ml of sterile distilled water for 20 minutes on an orbital shaker. Serial dilutions were prepared and 0.1 ml aliquot of 10^{-5} to 10^{-8} dilutions support coated on NB plates, followed by incubation at 28 ± 1 °C for 48 hours. Population powder formulations were recorded at 7, 15, 30, 60, 90 and 120 DAS. Following formula was used to determine the population dynamics in prepared formulation:

$$\text{(cfu) per gram of sample} = \frac{\text{mean (cfu) X dilution factor}}{\text{Quantity of sample at dry weight basis}}$$

Results and discussion

Effect of days after storage on different temperature

Data recorded on colony forming units (cfu) on regular intervals indicate that maximum population dynamics was recorded at 28 °C when counted at 7, 30, 60, 90 and 120 days after storage (DAS) rather than at 5 °C. An increasing trend in cfu was seen with the increase in time interval and maximum cfu (312.67 cfu) was recorded at 60 DAS in the treatments stored at 28 °C compared with (193.67 cfu) at 5 °C as given in Table 2. Decrease in population was observed at 90 DAS and lowest bacterial population was recorded at 120 DAS at both 5 °C and 28 °C. Data regarding population dynamics in each time interval at both the temperature levels is given (Tables 1-5).

Data on cfu for rhizobacterial cultured on two carrier materials at 5 °C and 28 °C was also compared. Results show that maximum colony count was recorded at 28 °C by *Bacillus subtilis* on Organic matter (BSOM) followed by *Bacillus subtilis* on Vermi compost (BSV) and *Pseudomonas fluorescens* on Organic matter (PFOM) 60 DAS as shown in Table 1-5.

Rhizobacteria showing the maximum biocontrol efficacy were multiplied on suitable carrier materials for the efficient release of the bacteria. For this mass culturing of rhizobacteria viz., *Pseudomonas fluorescens*and *Bacillus subtilis* was carried out on vermicompost and organic matter as (Omer, 2010) mass multiplied *Bacillus megaterium* on 21 different carrier materials and found Talc - glucose, Talc - yeast and Cellulose - clay based powder formulations and evaluated them for efficacy under open field conditions.

Table 1. Rhizobacteria colony forming units (CFU) on different carrier materials after 7 days of storage.

Treatment	Temperature	
	5 °C	28 °C
PFV	12.33	52.33
BSV	23	60.67
PFOM	20	78.33
BSOM	48	117.67

Table 2. Rhizobacteria colony forming units (CFU) on different carrier materials after 30 days of storage

Treatment	Temperature	
	5 °C	28 °C
PFV	103.6667	280.3333
BSV	169.67	293.67
PFOM	194.3333	311.6667
BSOM	219	372.3333

After formulating the biopesticides on carrier materials *viz.*, vermicompost and organic matter, population dynamics was studied for *Pseudomonas fluorescens* and *Bacillus subtilis* at 7, 30, 60, 90 and 120 days after storage (DAS) at 5 °C and 28 °C. Maximum cfu were measured at 28 °C at 60 DAS while decrease in population was noticed at 90 DAS. Results are in contrast with the findings of (Bazilah *et al.*, 2011) that studied the sustainability and effectiveness of *Burkholderia* sp. and *Pseudomonas* sp. at varying temperatures 10, 20 and 30°C after 9 months of storage.

Table 3. Rhizobacteria colony forming units (CFU) on different carrier materials after 60 days of storage

Treatment	Temperature	
	5 °C	28 °C
PFV	193.6667	312.6667
BSV	222.33	388
PFOM	264.33	389.6667
BSOM	345	515

Table 4. Rhizobacterial colony forming units (CFU) on different carrier materials after 90 days of storage

Treatment	Temperature	
	5 °C	28 °C
PFV	25.67	55
BSV	28	66
PFOM	35.67	76.67
BSOM	44.66	89.67

He found that dry formulations and liquid based formulations showed maximum population dynamics at 10 and 20 ºC after 9 months of storage. Formulations development is important for the efficient release of the biocontrol agents into the soil and formulation development is supported by the claim of (Sahni *et al.*, 2008) that cultured the beneficial *Pseudomonas syringae* on vermicompost to control soil borne diseases and reported that application of vermicompost based formulation enhance the plant growth and also controls the seedling mortality in chickpea.

Table 5. Rhizobacteria colony forming units (CFU) on different carrier materials after 120 days of storage.

Treatment	Temperature	
	5 ºC	28 ºC
PFV	4	10.33333
BSV	7.33	12.67
PFOM	9	19
BSOM	17	24.66667

The aim of this study is to optimize the nutritional value using different substrates to developed formulations with better shelf life and rhizobacterial viability for disease control and plant growth enhancement.

Fig. 1. Preparation and sterilization of different carrier material.

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