Original Research Article   https://doi.org/10.20546/ijcmas.2020.908.039

Evaluation of *Pseudomonas fluorescence* based Commercial Biopesticide Products against their Indicated Phytopathogens

Mehjebin Rahman*, P. K. Borah and L. C. Bora

Department of Plant Pathology, Assam Agricultural University, Jorhat-785013, Assam, India

*Corresponding author

A B S T R A C T

Keywords
Biocontrol agents, Inhibition percentage, Population count
Article Info
Accepted: 10 July 2020 *Available Online:* 10 August 2020

The present investigation was conducted to evaluate some *Pseudomonas fluorescence* based commercial biopesticides marketed in Assam. Total six biopesticide products were evaluated. Both qualitative and quantitative assessments of the biopesticide products were carried out during the study. Quantitative assessment of the products revealed the presence of indicated bioagents only in three products: Commercial Biopesticide Products (CBP)-1, Commercial Biopesticide Products (CBP)-2 and Commercial Biopesticide Products (CBP)-3 with population count of 1.3×10^9, 6.5×10^8, 8.0×10^8 cfu/ml or cfu/gm, respectively. The three *Pseudomonas fluorescens* isolates were further qualititatively assessed *in vitro* for evaluating their efficacy against their indicated plant pathogens. *P. fluorescens* from Commercial Biopesticide Products (CBP)-3 was found most effective against *Alternaria brassicae* (85.50%), from Commercial Biopesticide Products (CBP)-1 and Commercial Biopesticide Products (CBP)-2 against *Fusarium oxysporum* fsp *lycopersicum* (80.00%) and *Alternaria brassicae* (85.50%), respectively.

Introduction

Biopesticides are effective and biodegradable substance without any residues in the environment as compared to the synthetic pesticides which are the earliest and most common method used for controlling pests and diseases in plants. Biopesticides comprise only a small share (5.0 per cent) of the total crop protection market globally (Christos *et al.,* 2018) and 4.2 per cent of the entire pesticide market in India (Gautam *et al.,* 2018). Till recently, about 175 active ingredients and 700 products have been registered worldwide (Kachhawa, 2017). However, the use of biopesticides is increasing globally by approximately 10 per cent every year (Kumar and Singh, 2015). It seems that, if the global market increases further in the future, the biopesticides can play a vital role in substituting chemical pesticides reducing the current over-reliance on these ecologically harmful chemicals.

In India, since early nineties, microbial biopesticides have been produced by a good number of entrepreneurs which now counts to be around 400. But, only 10 per cent of these production units have proper facility and rest of the 90 per cent biopesticides enterprises are run for short term gains which are mostly 'one-man-shows'. The later, do not have good

facilities with regard to fermentation equipment, qualified microbiologists, trained staff, and sufficient space for operating each step of production, enough buffering capacity and rolling capital. Such enterprises with lack of proper infrastructure often end up with physical and biological contamination (Koul *et al.,* 2012).

It is very difficult to produce biopesticides with constant purity like synthetic pesticides, which are produced in desired purity and yield. A wide variation occurs in the active and associated ingredients of the products if mishandled during processing and transporting. These lead to variation in the chemical, physiochemical, phyto-compatibility, toxicological and other related properties of the products. However, the most important issue with the biopesticide products are contamination of the products with other microbial contaminants. The lack of proper standards and analytical procedures for manufacturing of these products and also poor shelf life of the bioagents in the products are the serious impediments in quality control of biopesticides (Isman, 2006). Quality control needs to be ensured at all levels of mass production of the nucleus culture (biocontrol agents) by taking extreme care in all the steps of production, i.e. isolation and preservation of the bioagents; storage, transportation and releasing techniques of the bioproducts.

In Assam, the scope of biopesticide industry can be visualized through the 'Act East Policy' develop by the Govt of India. The border between North-East India and South Asian countries will be turned into a trade and business hub of South East Asia. This seems to be a potential boon for North East Indian growers to enter into the South East Asian market for trading of agricultural commodities and processed food products most specifically of organic origin. Biopesticides in the Agro-markets of Assam

are manufactured by several commercial companies in different districts of the State. Besides, some biopesticide products are also imported from different states by these companies as per demand of the local growers.

Bacteria under genus *Pseudomonas* and *Bacillus* are the most promising biocontrol agents that have the abilities to act against wide range of phytopathogens. They suppress the disease causing activity of plant pathogenic microbes through several mechanisms like antibiosis, secretion of volatile toxic metabolites, mycolytic enzymes, parasitism and competition for food and space. *Pseudomonas* species, *P. fluorescens, P. putida, P. cepacia* produces antibiotics, *viz.,* Pyrol, Nitrin, Oomycin-A and hormones such as Indole acetic acid (IAA), Gibberillic acid (GA) and Siderophores, that inhibit the growth of pathogens (Bhattacharjee *et al.,* 2014).

Materials and Methods

Present investigation on *Pseudomonas fluorescens* biopesticides with special reference to evaluation of antagonistic activity of *P. fluorescens* against fungal pathogen were carried out in the Department of Plant Pathology, Assam Agricultural University, Jorhat (Assam).

For isolation, one gram of biopesticide sample was placed in a 250 ml conical flask containing 100 ml of sterilized distilled water (SDW) and mixed thoroughly. Different dilutions of working samples were prepared by serially diluting the stock solution upto 10^{-8}. One ml of last serial dilution *i.e.*, 10^{-8} was spread on *P. fluorescens* selective King's B Medium (King's *et al.,* 1954) for isolation of *P. fluorescens.* The plates were incubated for 2 days at 28 ± 2ºC and after incubation, pure culture was grown; colour of bacterial colony

was initially yellow but turned yellow green as pigmentation were produced (Bonds, 1957). The numbers of colonies formed on each medium were counted at 48-72 hrs for bacteria and at 120 hrs for fungi after incubation. Colony forming units (cfu) were calculated using the equation of Johnson and Case (2007).

$$\text{Colony forming Unit (Cfu)/gm or Cfu/ml} = \frac{\text{No of Colonies} \times \text{Dilution Factor}}{\text{Aliquot taken (ml)}}$$

The bioagents isolated were preserved for their further studies to evaluate the bioactive potential against plant pathogens.

Culture and maintenance

The medium consists of 20gm peptone, 20gm agar-agar, 1.5gm potassium monophosphate (K2HPO4), 1.5gm magnesium sulphate (MgSo4), glycerol 10mL and distilled water 1000 mL (King *et al.,* 1954)**.** All the ingredients were mixed with distilled water homogenously. The media was then placed into a stainless steel pan and stirred with glass rod for proper mixing of all the ingredients. Now the medium was filtered through a muslin cloth by squeezing out whole liquid. 200 ml medium was placed in each 500 ml capacity flasks. These were tightly plugged with non-absorbent cotton plug and wrapped with butter paper and rubber band. Medium was autoclaved at 15 psi (121.6ºC) for 20 min and cooled before pouring into Petri plates. Another medium contain agar–agar 20.0g, dextrose 20.0g, potato (peeled and sliced) 200.0g, distilled water 1l. 250g of potato was peeled and cut into small and fine sliced pieces. Exactly 200g of potato pieces were weighed and placed into a stainless steel pan. 500 ml of water was added to potato pieces and boiled gently for such a period until they were easily mashed by a glass rod. The decoction was filtered through muslin cloth and squeezed out all the liquid in a measuring cylinder and potato pieces were discarded.

Now, sufficient amount of water was added to make the volume 1000 ml. The preweighed agar agar was added (20g) bit by bit to the boiling solution to dissolve it. At the same time dextrose (20g) was also added in boiling solution (melted with agar) and final volume made up to 1 l. It was poured at about 200 ml in each of four conical flasks of 500ml and 10 ml per culture tube to prepare the PDA slants. Both, flasks and culture tubes were tightly plugged with nonabsorbent cotton and wrapped with butter paper and rubber bands.

The culture tubes and flasks were placed vertically (mouths up) in wire baskets and then autoclaved at 15 psi (121.6ºC) for 20 min. The bacteria, initially isolated in a pure culture on King's B media and sub cultured on PDA slants were allowed to grow at 28±20°C temperature. The culture thus obtained was stored in refrigerator at 50°C for further studies and was sub cultured periodically.

Antagonism of *P. fluorescens*

The antagonistic activity of *P. Fluorescens* against Foc, *Alternaria* sp., *Colletotrichum* sp,. *Rhizoctonia solani* and *Sclerotium rolfsii* were tested by dual culture technique. For evaluation of *P. fluorescens* inhibition efficacy against the fungal pathogens, 5 µl of bacterial suspension (10^8 cfu/ml) was placed on the four sides of the plate. After 48 hrs incubation at 28 ± 2°C, a 6 mm diameter mycelial disc was placed at the center of plates. Then, plates were incubated at 28±1°C for 7 days. The growth diameter of the pathogen (distance between the point of placement of fungal disk and actively growing edges of the fungus) was measured. The percentage of growth inhibition was calculated according to Erdogan and Benlioglu (2010) method. This experiment was conducted thrice.

Per cent inhibition (I) = C-T/C ×100

Where,

C- mycelial growth of pathogen in control
T- mycelial growth of pathogen in dual culture plate.

Results and Discussion

Isolation of *P. fluorescens*

Six biopesticides indicating the presence of *P. fluorescens* were assessed. On the basis of colony characteristics and pigmentation etc, only three isolates were confirmed to be *P. fluorescens* isolated from three biopesticide products and tested against *Fusarium oxysporum* f.sp. *lycopersici, Rhizoctonia solani,* and *Alternaria brassicae, Sclerotium rolfsii, Colletotrichum gloesporoides*.

The three *Pseudomonas fluorescens* isolates isolated and confirmed as *Pseudomonas fluorescens* during present investigation were designated as given below:

Pseudomonas fluorescens isolates	Biopesticide Product
Pf 1	Commercial Biopesticide Products (CBP)-3
Pf2	Commercial Biopesticide Products (CBP)-1
Pf3	Commercial Biopesticide Products (CBP)-2

Dual culture technique

The results of the dual culture technique indicated that the three isolates inhibited growth of tested fungi significantly. In case of *Fusarium oxysporum* f.sp. *lycopersici* a maximum inhibition of 84.80% was recorded by Commercial Biopesticide Products (CBP)-2 and minimum of 76.20% was recorded with the isolate Commercial Biopesticide Products (CBP)-3. In case of *Alternaria brassicae*, the maximum inhibition of 85.50% was exhibited by Commercial Biopesticide Products (CBP)-

2 07 and minimum, 70.20% was recorded with the Commercial Biopesticide Products (CBP)-1 isolate, where as in case of *Colletotrichum gloeosporoides* maximum inhibition 84.80% was recorded by Commercial Biopesticide Products (CBP)-2 and minimum, 63.70% was recorded with isolate Commercial Biopesticide Products (CBP)-3. Again, in case of *Rhizoctonia solani* and *Sclerotium rolfsii* highest inhibition was recorded to be 40.00% and 77.70% respectively by Commercial Biopesticide Products (CBP)-2 and lowest was recorded 26.00% and 60.00% respectively by Commercial Biopesticide Products (CBP)-3. (Plate 2, Table 2, Fig 1).

With the aim to help the local growers and farmers of Assam to facilitate with quality bio inputs which in turn may augment in their strategy of organic conversion of their farmland and products, and also to help them in exporting their quality proven products, the present research programme has been conceived. In this direction six *Pseudomonas fluorescens* biopesticides were isolated on King's B medium and further they were subjected to identification on the basis of antagonistic characteristics exhibited against some pathogenic microflora. Out of six biopesticides isolated on King's B medium, only three were confirmed as *Pseudomonas fluorescens.* The isolate Pf1 was isolated from Commercial Biopesticide Products (CBP)-3 biopesticide product while the isolate Pf2 was isolated from Commercial Biopesticide Products (CBP)-1 biopesticide product of Tropical Agro and isolate Pf3 from Commercial Biopesticide Products (CBP)-2.

A similar work was also done by All India Co-ordinated Project on Biological Control, Bangaluru and they have also reported that a very low level of active bioagents wrre present in the tested commercial biopesticide products.

Table.1 Quantitative characteristics of evaluated biopesticide products

Name of products	Active bioagents	Population (cfu/ml or gm)
Commercial Biopesticide Products (CBP)-1	*Pseudomonas fluorescens*	1.3×10^9
Commercial Biopesticide Products (CBP)-2	*Pseudomonas fluorescens*	6.5×10^8
Commercial Biopesticide Products (CBP)-3	*Pseudomonas fluorescens*	8.0×10^8

Fig.1 Comparative efficacy of different biopesticides against the targeted pathogens
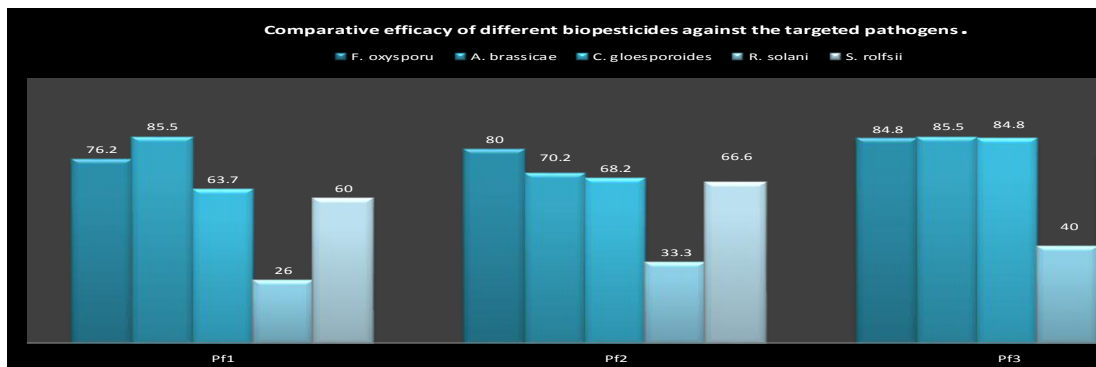

Plate.1(a-c) Colony forming units of *Pseudomonas fluorescens* from a) Commercial Biopesticide Products (CBP)-1, b)Commercial Biopesticide Products (CBP)-3, c) Commercial Biopesticide Products (CBP)-2
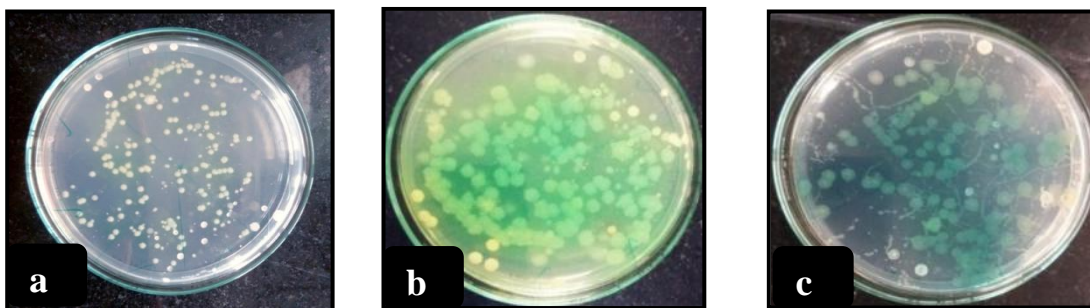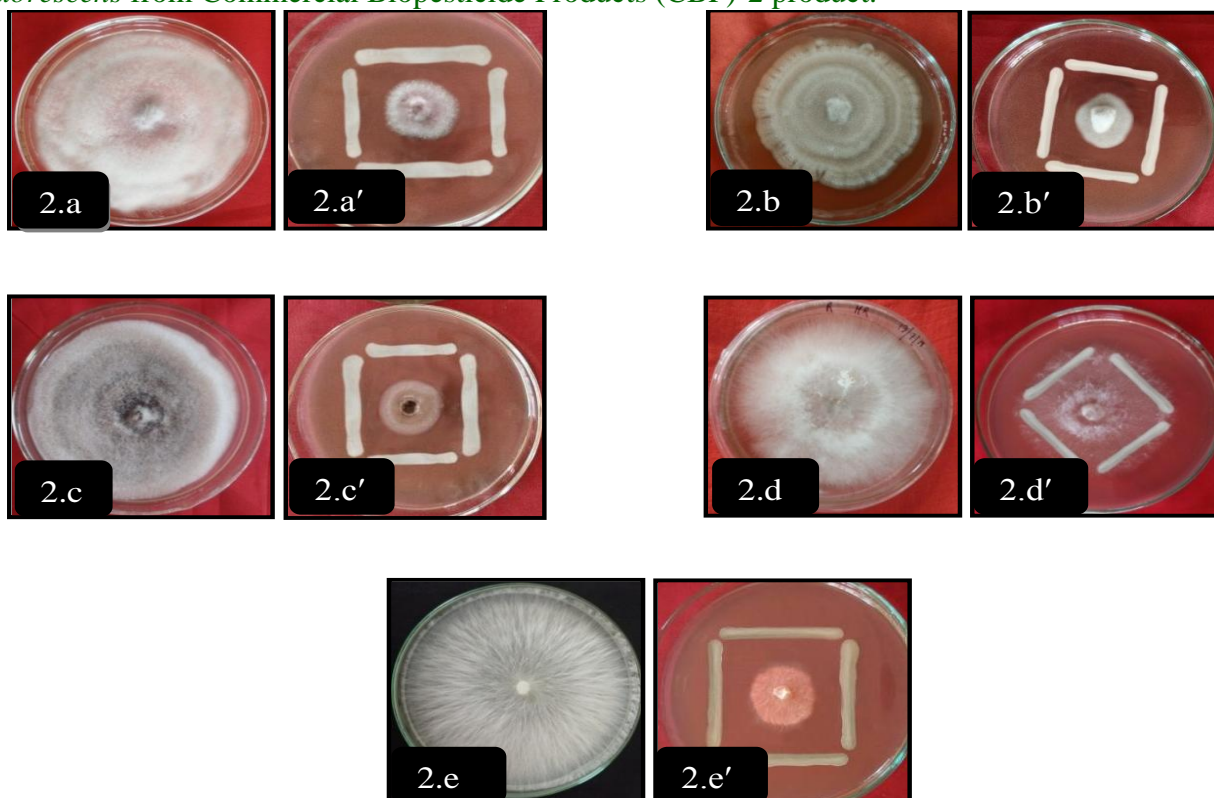
Table.2 Antagonistic activity of *Pseudomonas fluorescens* against indicated fungi

Pseudomonas fluorescens Isolates	Test Fungi	Mycelial growth (mm)	Growth inhibition (%)
Pf1	*Fusarium oxysporum* fsp. *lycopersici*	10.70	76.2 (60.80)
Pf2		9.00	80.0 (63.43)
Pf3		6.80	84.8 (67.05)
S.Ed (±)			0.32
CD$_{0.05}$			0.66
Pf1	*Alternaria brassicae*	14.70	85.5 (67.72)
Pf2		13.4	70.2 (56.91)
Pf3		6.50	85.5 (67.62)
S.Ed (±)			0.59
CD$_{0.05}$			1.22
Pf1	*Colletotrichum gloesporoides*	16.30	63.7 (52.95)
Pf2		14.30	68.2 (55.67)
Pf3		6.80	84.8 (67.05)
S.Ed (±)			0.46
CD$_{0.05}$			0.95
Pf1	*Rhizoctonia solani*	33.00	26.0 (30.66)
Pf2		30.00	33.3 (35.24)
Pf3		27.00	40.0 (39.23)
S.Ed (±)			1.94
CD$_{0.05}$			4.01
Pf1	*Sclerotium rolfsi*	18.00	60.0 (50.77)
Pf2		15.00	66.6 (54.70)
Pf3		10.00	77.7 (61.82)
S.Ed (±)			1.08
CD$_{0.05}$			2.23

( ): Arcsine transformation value

Plate.2 a) Control plate of *F. oxysporum* f.sp. *lycopersici*, b) Control plate of *A. brassicae*, c) Control plate of *C. gloeosporoides*, d) Control plate of *R. solani*, e) Control plate of *S. rolfsii*, a′) *F. oxysporum* f.sp. *lycopersici* in presence of *P. fluorescens* from Commercial Biopesticide Products (CBP)-2 product, b′) *A. brassicae* in presence of *P. fluorescens* from Commercial Biopesticide Products (CBP)-2 product, c′) *C. gloeosporoides* in presence of *P. fluorescens* from Commercial Biopesticide Products (CBP)-2 product, d′) *R. solani* in presence of *P. fluorescens* from Commercial Biopesticide Products (CBP)-2 product, e′) *S. rolfsii* in presence of *P. fluorescens* from Commercial Biopesticide Products (CBP)-2 product.

The results of the dual culture technique indicated that the three isolates were able to inhibit growth of tested fungal pathogen significantly. In case of *F. oxysporum* f.sp *lycopersici* a maximum and minimum inhibition was exhibited by Pf3 (84.80%) and Pf1 (76.20%) respectively. In case of *Alternaria brassicae*, the maximum inhibition of 85.50% was exhibited by Pf1 and Pf3 and minimum, 70.20% was exhibited by Pf2 where as in case of *Colletotrichum gloesporoides* maximum inhibition 84.80% was exhibited by Pf3 and minimum, 63.70% was exhibited with isolate Pf1. Again in case of *Rhizoctonia solani* and *Sclerotium rolfsii*

the maximum inhibition were 40.00% and 77.70% respectively exhibited by Pf3 and minimum were 26.00% and 60.00% by Pf1 respectively. Wagner *et al.,* (2018) suggested that fluorescent pseudomonads had antagonistic effect that was not necessarily associated with the production of antibiotics. Several reports on plant-associated pseudomonads indicated also a role of siderophores, lytic enzymes, hydrogen cyanide and ammonia as well as organic volatiles in the inhibition of fungal phytopathogens.

From the present study, it is found that most

of the biopesticide products do not retain the indicated bioagents. Some of them also contained microbial contamination at a high amount. A good infrastructure, a proper supply chain for nucleus cultures and a stable ecosystem with required temperature and humidity should be established to maintain the quality of products. A number of characteristics are desirable for the biocontrol agent to become commercial reality. Strong synergies between Ministry of Agriculture, Govt of India, CIPMCs, State Agriculture Departments, SAUs and ICAR Institutes, Private Biopesticides Industries, KVKs, NGOs etc. need to be developed to attain the overall goal of promotion of biopesticides. Certain quality issues like the original and true to type of biocontrol strains, efficacy, minimum spore concentration, physical parameters of formulated products and free from contaminants and chemical pesticides are of paramount importance for the registration of biopesticides. The quality of biopesticides should not be compromised at any cost during production and storage for ensuring the supply of authenticated products to the farmers.

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