A Novel Biopesticide Formulation for Environmental Sustainability

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ABSTRACT: Increasing rate of environmental pollution due to extensive use of chemical pesticide in agricultural sector to prevent crop infestation is a vital matter of concern. Biopesticides offer solutions to pest resistance replacing conventional chemical pesticides. Our integral vision was to formulate a product which satisfies all the three criteria of being cost effective, eco-friendly and easy availability. We have used a combination of 5 microorganisms among which *Trichoderma viride*, *Beauveria bassiana, Paecilomyces lilacinus*, are entamopathogenic fungi and *Bacillus thuringiensis* and *Bacillus subtilis* potent insecticide, fungicide as well as plant growth promoters. Application of the formulated products on *Capsicum annum*, ensured protection against pest infestation and also exhibited enhanced plant growth with longer shelf life and long run retention capacity in phyllo as well as rhizosphere and being completely organic and biodegradable in nature promotes environmental sustainability.

Keywords: *Environment,, Biopesticide, Chemical pesticide, Entamopathogenic fungi, Plant growth promoter*

1.Introduction

Biopesticides, used to suppress pest population, consists of living organisms or products derived from these organisms. Biopesticides decompose more quickly in the environment and are generally less toxic towards non- specific target species [12]. Thus they can often help suppress resistant pathogens and can be applied in alternation with other pesticides. Biopesticides generally contains low volatile organic chemicals (VOC) content and thus reduces air pollution caused by high-VOC chemicals compared to chemical pesticides. In other words, these beneficial microorganisms are generally obtained from aerial or underground parts of plants that are naturally less or not at all affected by a pathogen that devastates a neighboring group of the same plant species [2]. Using microorganisms which are a part of our biome as pesticide eliminates the need to incorporate any chemical substance in the ecosystem and thus doesn't disturb the ecological balance. The use of non- degradable chemicals in farming is harmful as it accumulates in tropic levels, and disturbs the balance of the ecosystems. Phyto-stimulation is a direct promotion of plant growth through the modulation of the plant's hormonal balance. Several microorganisms are capable to produce and excrete a variety of plant hormone-like compounds including auxin, gibberellins, cytokines etc. and also enzymes that degrade a precursor of ethylene thus promoting plant growth especially under stress conditions. Biopesticides are effective in very small quantities and can decompose quickly, largely avoiding the pollution problems that are caused by conventional chemical pesticides, when used as a component of Integrated Pest Management (IPM) programs, can greatly decrease the use of conventional pesticides, without decreasing the crop yields. Moreover, a microorganism that possesses a combination of these growth-promoting activities and bio control potential offers the advantage to supply the crop in one application with a bio pesticide and a bio fertilizer.

1.1. Objectives

- To propose a novel process for producing biopesticides in liquid as well as solid phase (none powder form) using non- pathogenic microbes that support the survival as well as multiplication of the bio agents during storage at room temperature and marketing with carrier at a low cost.

- To check the level of interaction among the organisms considered for use

1.2. Microorganisms with beneficial bio-control properties selected

- *Bacillus thuringiensis* a ubiquitous gram-positive, spore-forming bacterium that forms parasporal crystal during the stationary phase of its growth cycle responsible for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera. There are more recent reports of *B. Thuringiensis* isolates active against other insect orders (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and against nematodes, mites, and protozoa.[3] They are also found to produce fungicidal enzyme chitinase

- *Bacillus subtilis*, possess several characteristics that enhance its survival in the rhizosphere and is also found to be effective as a biopesticide. They can display various mechanisms of biocontrol [8] and biostimulation (produces auxin) and known to secrete chitinase which inhibits pathogenic fungal growth in rhizosphere. [4]

- *Beauveria bassiana* produce some secondary metabolites in which beauvericin have insecticidal activity, cytotoxic and ionophoric property. It parasitizes a wide range of arthropods Such as termite, thrips, aphids, whiteflies, rice pests, corn borer, sucking pests and different types of beetles. [1] [7]

- *Trichoderma viride* colonizes in root and produces growth promoter gibberellins. They also secrete cellulase, glucanase and other enzyme which can dissolve the hyphae of invading mycelial fungi, thus reduce their virulence and the plants are protected from harmful fungus. [8]

- *Paecilomyces lilacinus*, a common soil hypomycetes with a cosmopolitan distribution, parasitizes eggs of root knot causing agents such as nematode *Meliodogyne incognita*, Chitwood, *Macrophomina phaseolina* etc.[11]

2. Materials and methodology:

2.1. Revival of organism:

1 gram of powdered sample of each organism was suspended in 10 ml of distilled sterile water and .1 ml of each of them were plated and revived after 48 hour incubation.

2.2. Isolation and Purification of culture:

Suitable colony from the above incubated plates were taken and streaked on PDA (for fungi) and NA (for bacteria) slants then incubated for 24 hours.

2.3. Characterization of the organism:

Bacteria from the pure culture were subjected to Gram staining and fungi from the pure culture was stained with lacto-phenol cotton blue stain and observed under light microscope.

2.4. Interaction Study:

All possible combinations between bacteria-bacteria, fungi-bacteria as well as fungi- fungi were studied to check to determine their interaction behavior. In case of bacteria and fungi, the growth was checked on both Nutrient agar as well as Potato dextrose agar (PDA).

2.4.1. *Fungal- Bacterial interaction-*

With the help of a sterilized cork borer, disc was cut out from PDA plates containing fungal pure culture and was placed in the center of the plate with bacterium streaked along four sides of the plate, which were then allowed to incubate at 37^0 C and 25^0 C in duplicate sets for 48 hrs.

2.4.2. *Fungal- Fungal interaction-*

With the help of separate sterilized cork borers, discs were cut out from plated containing pure culture and then the two fungal discs were placed equidistant from the central line of the PDA plate and incubate at 25^0 C in duplicates for 72 hrs.

2.4.3. *Bacterial- Bacterial interaction-*

Two individual bacteria from pure culture were T-streaked on NA plates and allowed to incubate at 37^0 C for 24 hrs.

2.4.1. Preparation of soil for experimental set up:

24 pots of soil were prepared as follows:
Vermicompost: 600 gm. /kg + *Sesbania*: 200 gm. /kg + PSB: 0.5 gm. /kg + KSB: 0.5 gm. /kg (table1)

6 pots were dedicated for each experimental set namely control base soil, control chemical pesticide, SXCPB1, SXCFP1, SXCFP2 and SXCFP3.

2.5. Formulation of liquid pesticides:

2.5.1. *For bacterial pesticide* **(SXCPB1)**:

1% peptone and 0.5% dextrose was added in sterile distilled water followed by addition of 1.5% rice bran oil to prepare an emulsion. Then *Bacillus thuringiensis* and *Bacillus subtillis* spore suspension was inoculated in 1:1 ratio. The formulation was incubated at 37°C for 24 hours.

2.5.2. *For fungal pesticide 1* **(SXCPF1)**:

2% rice bran oil was added to potato decoction to prepare an emulsion followed by the addition of 10% *Beauveria bassiana* spore suspension and 72hrs incubation at 25°C.

2.5.3. *For fungal pesticide 2* **(SXCPF2)**:

2% rice bran oil was added to potato decoction and mixed well to prepare an emulsion, 5% then 1:1 *Trichoderma viride* and *Paecilomyces lilacinus* spore suspension was added and incubated at 25° C for 48 hrs**.**

2.5.4. *For fungal pesticide 3* **(SXCPF3)**:

A mixture of charcoal powder and oil cake was made in 3:7 proportion followed by addition of 2% w/w, 1:1 *Trichoderma viride* and *Paecilomyces lilacinus* crushed mat and thorough mixing. The formulation was then packed in zip- lock bags and incubated at 25° C for 48 hrs.

2.6. Application of formulations on plant set ups:

After 2 weeks of germination, products were applied in desired amount to the plants accordingly. 6 pots of control plants with base soil were only watered without any supplementary addition and the other 6 control pots were supplemented with renowned chemical pesticide. 0.8g of SXCFP3 was added in rhizosphere of the experimental set at a regular interval of 2 weeks, to prevent soil borne pathogenic attack of the plants. And of the other three types of formulation (SXCBP1, SXCFP1, SXCFP2) were sprayed on emergence of aphids or flies. *Capsicum annum* was selected as model plant to determine effects of biopesticide application, due to its fast growth rate and suitability to climate of Eastern India.

2.7. Chlorophyll assay:

Acetone extract of 10g fresh leaves from each set up was centrifuged at 5000 rpm for 5minutes in room temperature and absorbance of chlorophyll content was measured at 645nm and 663 nm using UV-Vis spectrophotometer [10].

2.8. Shelf life check:

The shelf life of formulated products were checked after 3 months of formulation by spread plate technique.

3.Results and observations

3.1. Interaction Study:

All possible combinations between bacteria-bacteria, fungi-bacteria as well as fungi- fungi were studied to check for their interaction and growth of the organisms were observed (table 2) as follows:

- Partial positive interaction between *Beauveria bassiana* and *Trichoderma viride* was observed. Though they were not inhibiting each other's growth, but much prominent growth was not observed.
- Positive interaction between *Trichoderma viride* and *Paecilomyces lilacinus* was observed. Their growth was better as compared to the growth observed in case of the interaction of *Beauveria bassiana* and *Trichoderma viride*. In both the interactions, there was no dominance of one genus over the other.
- *Paecilomyces lilacinus* and *Beauveria bassiana* though not antagonistic showed obscure growth in collaboration.
- A mutual growth pattern was observed in between *Bacillus subtilis* and *Bacillus thuringiensis.*
- *Bacillus (*both *thuringiensis & subtilis)* were observed to inhibit growth of all the fungus selected for interaction studies. (This might be because of *Bacillus* genus's known capacity to produces chitinase enzyme, which inhibits fungal growth by destroying their cell wall structural unit- chitin.[6] (Wang et al. 2002))

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3.2. Growth measurement of host:

After sowing of the seeds in potted soil, before application of product following observations were recorded (table 3).
After application of formulated product developmental of the plants- root length (Fig3) and shoot length (Fig4) were measured. The plant setups were observed to get prevented from pest and no negative impact on their growth or death was observed. All the sets were growing green and healthy. Moreover the growth promoting factors produced by *B. subtilis* such as Auxin [13]. and by other members promoted increased shoot length and root length in SXCBP1 applied plants compared to other set ups where increased shoot length, root length (Fig 3) was not observed in a drastic level but a gradual increase similar to the control was noted (Fig2)

3.3. Chlorophyll assay:

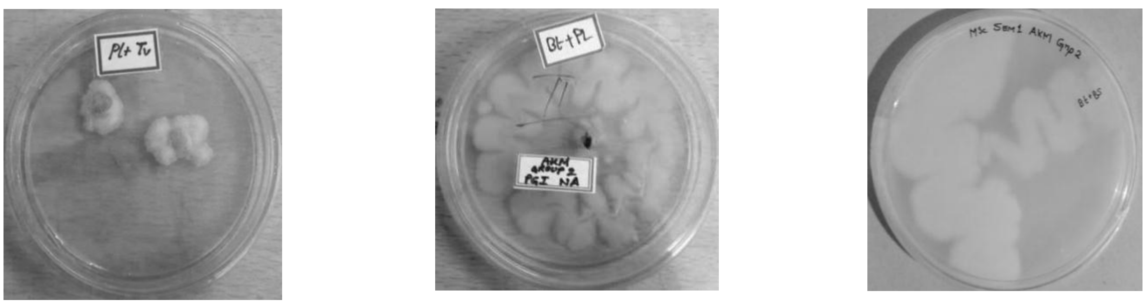
Chlorophyll assay of the experimental hosts were done to check developmental status. Chlorophyll assay was performed samples were spectroscopically measured [10] (Fig 5).
It was observed that the chl a: chl b ratio for all the treated plants lay nearby the expected range. Especially in case of SXCBP1 applied plants, quite a good level of chlorophyll content in the host plants was observed.

3.4. Shelf life check:

On checking after 3 months, SXCBP1 was $2X10^8$ CFUs/litre formulation, SXCFP1 was $1X10^7$spore/ml and SXCPF2 was $6X10^9$ spore/ml.

4. Tables, Figures and Equations

4.1.Interaction study



(i) (ii) (iii)

Fig 1: Interaction study between (i) *Trichoderma viride* and *Paecilomyces lilacinus (ii) Bacillus thuringiensis* and *Paecilomyces lilacinus* (iii) *Bacillus thuringiensis* and *Bacillus subtilis*

5.2.Observation for growth parameters after application of formulations

1.5 months`	**3 months**		**1.5 months**	**3 months**
				
Control base soil			Control with chemical pesticide	

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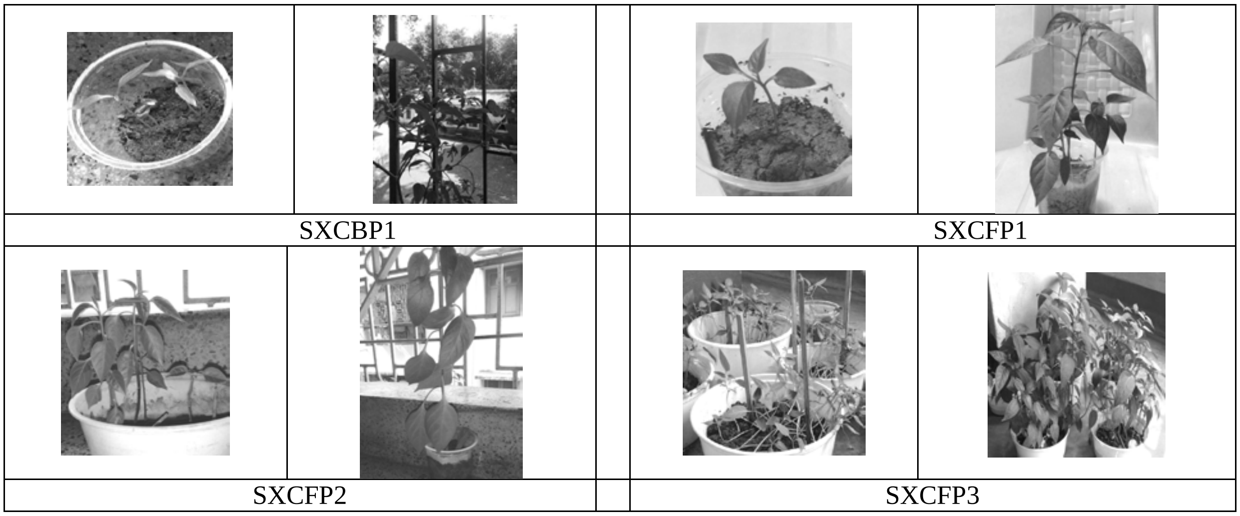

Fig 2: Experimental set up after application of formulated pesticide

5.3. Graph representing effects of formulation on root and shoot growth of the plants

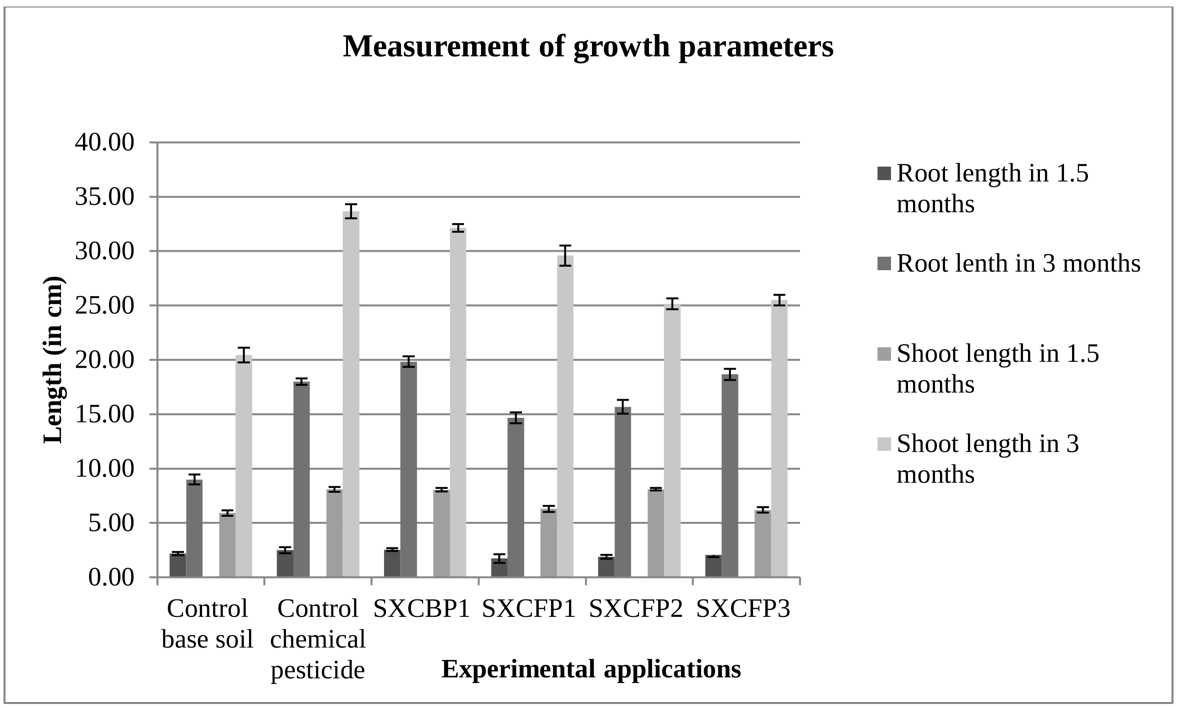

Fig 3: Graph representing comparative analysis of root and shoot length growth between 1.5 to 3 months after application of the formulations

5.4. Graph representing effects of formulation on chlorophyll content of the plants

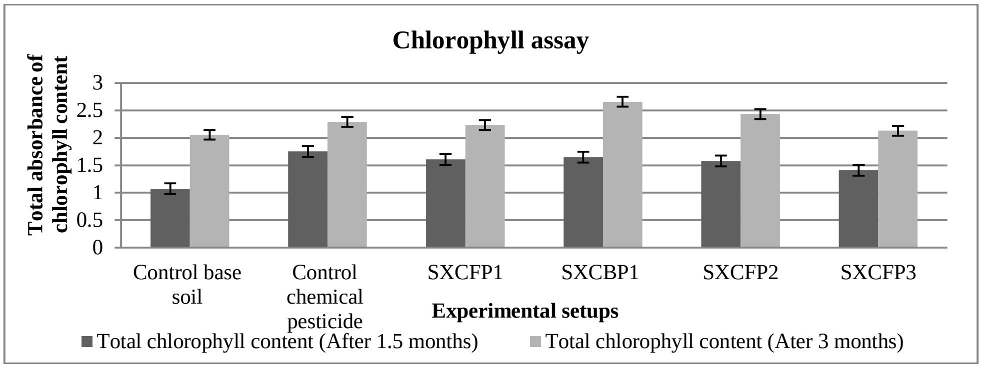

Fig 5: Represents the results for chlorophyll assay

5.5. Table 1: Properties of soil

Property of soil used	**Measurement**
pH	6.5
Electric conductance	450.5
Colour and texture	Greyish brown, clayey, sticky and lumpy

5.6. Table 2: Interaction Study

Interacting microorganism	**results**	**Interacting microorganisms**	**results**
B. bassiana– T. viride	+	*P. lilacinus- B. subtilis*	-
B. bassiana- B. subtilis	-	*P. lilacinus- B. thuringiensis*	-
B. bassiana - B. thuringiensis	-	*B. thuringiensis – T. viride*	-
B. bassiana - P. lilacinus	-	*B. thuringiensis – B. subtilis*	+
P. lilacinus- T. viride	+	*T. viride – B. subtilis*	-

(+) means that the organisms doesn't inhibit each other;
(-) means that the organisms are antagonistic
This indicates that the non- inhibitors can be put together in a single formulation.

5.7. Table 3: Host developmental check

Germination frequency	16/30	
Germination time of seeds	3 days	
Growth measured after 1.5 month	**Root length(average)**	**Shoot length (average)**
	3 ± 0.01cm	7±0.02cm

5. CONCLUSION

Use of biopesticide is quite unpopular in our country as it doesn't provide instant effects and mostly available in powdered form which shows low shelf life as well as retention capacity. This formulation not only manages and enhances diversity, by incorporating biological principles and resources into farming systems, but also intensify agricultural production in a sustainable manner compared to chemical pesticides, resulting in environmental as well as economic progress [9]. Biopesticide applications help farmers transit from highly toxic traditional chemical pesticides, into an era of truly sustainable agriculture. The formulation was sent for field trial and on application 70% positive response was observed. Additionally, increase in shoot and root length, chlorophyll content etc. was indicative of good health of the plants. Chemical pesticides are often reported to cause health hazards as well as infertility of soil if added in higher concentration than recommended but in a similar case, biopesticides being natural in origin doesn't cause any harm to the environment, and any effect caused due to it can be resolved in simpler ways [5]. Mostly they are not accepted due to low shelf life, less efficiency and reduced crop yield, but this formulation overcomes most of those flaws. Moreover, the production cost being quite cheap and the formulation being completely organic are of considerable interest to farmers. This biopesticide comprising non-pathogenic microorganisms are host specific with no impact on the environment and are not harmful to human or plants neither cause any soil or air pollution hence is the best option to choose for sustainable and a green future.

Application of all products, at different time intervals on different plant set ups, provide protection from pest and the growth promoting factors produced such as auxin by *B. subtilis* and gibberellins by *Trichoderma* promoted enhancement in root length and shoot length respectively. Chlorophyll content of the applied plants compared to non- treated ones was better. The shelf life was also checked after 3 months and was found to be retained. And moreover the product formulated was cost effective so will be affordable for all peasants to use. Therefore, the formulations can be effective in large scales in agricultural lands to gain healthy yield of crops by reducing plant morbidity due to pathogenic attack as well as reducing risk of chemical pollution of the surface and underground water. Hence, these formulations will be a solution to erode pollution caused by chemical pesticides.

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