DEVELOPMENT OF A NOVEL MICROBIAL CONSORTIA TO PRESERVE AND PROTECT THE RHIZOSPHERE OF SOIL AND FACILITATE ORGANIC AGRICULTURE

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Abstract: The shift to organic agricultural practices in the recent years has encouraged researchers to develop biological agents to combat the problems of pest infestation and the accumulation of chemicals in the soil. A number of microorganisms have been found to produce and regulate phytohormones which play a major role in the plant growth promotion, development and response to environmental changes including stress. Hence, our main target was to develop a phytohormone-based plant growth promoting agent to study the difference in plant growth and development after its application in the rhizosphere of soil, thereby determining the efficacy of the product. An interaction study was conducted among the three commonly available laboratory strains of *Bacillus megaterium, Bacillus thuringiensis* and *Trichoderma viride* and the approach was exploited for the formulation of two organic products. An increase in shoot length by 10%, increase in number of nodes by 6% and increase in the number of leaves by 23% were observed after the application of the fungal formulation. The chlorophyll content assay showed increased chlorophyll content in the leaves of plants after the application of the bacterial formulation. Higher disease resistance was observed too after the application of fungal product. The novelty of this approach lies not only in the formulations which resulted in growth promotion, protection of the plant against pathogens and longer shelf life but also in low capital costs, easy availability of carrier materials and the whole approach being environment friendly.

Index terms: Phytohormone, interaction study

1 INTRODUCTION

The human population has been found to increase with every passing year. The world needs to begin to greatly increase agricultural productivity to feed this growing population. More importantly, this has to be achieved in a sustainable and environmentally friendly manner. The first step in this direction would require abstaining from popular practices of using chemical based fertilizers and growth promoters. The soil microflora exists in complex ecological relationships with plant roots. These majorly include fungi and bacterial populations. A number of these microorganisms have been found to either produce or regulate phytohormones which play a major role in plant growth promotion. Plant Growth Promoting Bacteria (PGPB) may promote plant growth directly, usually by either facilitating resource acquisition or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogenic agents on plant growth and development, that is, by acting as biocontrol bacteria (Glick 1995). Plant hormones play key roles in plant growth and development and in the response of plants to their environment (Salamone et al. 2001). Moreover, during its lifetime, a plant is often subjected to a number of nonlethal stresses that can limit its growth until either the stress is removed or the plant is able to adjust its metabolism to overcome the effects of the stress (Nieto, Frankenberger Jr. 1989). Rhizosphere microorganisms may also produce or modulate phytohormones under in vitro conditions (Salamone et al. 2001) so that many PGPB can alter phytohormone levels and thereby affect the plant's hormonal balance and its response to stress (Nieto, Frankenberger Jr. 1989). Hence, to develop a phytohormone-based plant growth promoting agent, deep understanding of each hormone was required. It is

now well known that a wide variety of bacterial species produce phytohormones like auxins, gibberellins and cytokinins. For example, some strains of *Azotobacter* spp., *Rhizobium* spp., *Pseudomonas fluorescens, Bacillus subtilis, Paenibacillus polymyxa* have been found to produce cytokinin. However, the regulation of these plant hormone-producing bacteria is not clearly understood. IAA synthesized by bacteria like *Bacillus megaterium* is involved at different levels in plant-bacterial interactions. It provides the plant greater access to soil nutrients.

The application of chemical inputs such as fertilizers and pesticides has long been used to improve productivity in conventional agriculture. However, there is now a growing desire for alternatives to this system (Mark et al. 2006). There has been an enhancement in the development of biofertilizers and biopesticides to facilitate organic agriculture in different parts of the world. One of the major problems faced due to the application of certain biological agents was an increase in premature falling off of flowers from the plant thereby hindering efficient fruit formation resulting in decreased yield of the crop variety. Our aim was to formulate a product which would enhance considerable growth of the plant along with prevention of premature senescence of flowering buds. The formulations were designed so as to incorporate three plant hormone-producing microbial species, which are *Bacillus megaterium, Bacillus thuringiensis* and *Trichoderma viride. B. megaterium* has been found to produce cytokinin as stated by Castro et al. (2008). Chagas Jr. et al. (2015) mentioned that *B. thuringiensis* is a producer of auxins. *T. viride* is a rich source of gibberellins. The success and commercialization of plant growth promoting preparations depend on the linkages between the scientific organizations and industries. Commercial success of these formulations require economical and viable market demand, consistent and broad spectrum action, safety and stability, longer shelf life, low capital costs and easy availability of carrier materials. Our primary objective was to isolate and characterize possible strains of plant growth promoting microorganisms, develop a novel plant growth promoting product for efficient growth and fruiting of agriculturally significant plants and to test the developed product for its ideal functionality.

2. MATERIALS AND METHODS

2.1 *Microorganisms, media and culture conditions*

Commercially available strains of *Bacillus megaterium*, *Bacillus thuringiensis* and *Trichoderma viride* were used to develop the plant growth promoting formulation. These strains were obtained from Nimpith, Ramkrishna Mission. Pure cultures of the bacterial and fungal strains were maintained on Nutrient Agar and Potato Dextrose Agar. Prior to this step, lyophilized cultures were serially diluted up to dilutions of 10^{-3}. The dilutions were plated on to the respective media. The bacterial cultures were incubated overnight at 37°C whereas fungal cultures for 4-5 days at 25°C following which, growth was observed.

2.2 *Characterisation of isolates*

2.2.1 Bacterial culture

The morphological characteristics of the isolates were studied and Gram character was determined. The Colony Forming Units (CFU/ml) were calculated to measure the growth density. Biochemical tests (IMViC test, Catalase test) were performed to identify the bacterial strains using their chemical characteristics.

2.2.2 Fungal culture

Lacto phenol cotton blue staining was performed to study the morphological characteristics. The spore count was determined using haemocytometer.

2.3 Interaction study among the involved microorganisms

The interaction studies were performed to determine any possible interactions (antagonistic or neutral) among the members of the microbial consortia used in developing the formulation.

Interaction studies on NA and PDA, between:

a. *B.megaterium* and *T.viride*
b. *B.thuringiensis* and *T.viride*
c. *B. megaterium* and *B. thuringiensis*

The bacterial and fungal cultures were co-cultivated on Nutrient agar and Potato Dextrose Agar respectively and their growth was observed. In addition to this, interaction between the bacterial strains was studied by cross streaking method on Nutrient Agar.

2.4 Study of Soil Characteristics

A comparative study of soil characteristics was carried out between normal (garden) soil and treated soil (activated).

Composition of the treated (activated) soil: Normal soil, Vermi compost [60%], Sesbania [20%], Phosphate solubilising bacteria (PSB) [0.5%], Potassium solubilising bacteria (KSB) [0.5%].

Four tests were performed for the characterisation of the Activated and Normal soil. The pH, Water holding capacity, Electrical conductance and the microbial load of the soil were determined.

2.5 *Formulation of the novel organic growth promoting product*:

Based on the interaction studies of the involved microorganisms in the consortia, two formulations were developed, which were applied to the rhizosphere of the test plant. The test plant chosen was *Solanum lycopersicum* (tomato).

2.5.1 Composition of the bacterial formulation (liquid):

Nutrient Broth (NB) was inoculated with *Bacillus megaterium* and *Bacillus thuringiensis* and incubated at 37°C for 4 days. The composition of the formulation included Sunflower oil [1.5%], Dextrose [0.5%], Peptone [1%] and sterile distilled water. 50ml of cultures of *Bacillus megaterium* and *Bacillus thuringiensis* respectively were added. At the time of preparation of the formulation, the CFU/ml of *Bacilllus megaterium* at 10^{-1} and 10^{-2} dilutions were 1.00×10^{5} and 5.16×10^{5} respectively wherein the CFU/ml of *Bacillus thuringiensis* at 10^{-1} and 10^{-2} dilutions were 8.40×10^{4} and 3.76×10^{5} respectively.

2.5.2 Composition of the fungal formulation (solid):

Potato Dextrose Broth (PDB) was inoculated with *Trichoderma viride* and incubated for 10 days. Dry weight and wet weight of the fungal mat was measured. The composition of the formulation included Charcoal, Sawdust and Maize meal in the ratio of 1:5:5. 1.2g/kg of Trichoderma mycelial mat was added to the formulation accordingly. At the time of preparation of the formulation, the spore count in per ml unit of inoculum of *Trichoderma viride* was 2×10^{7}.

2.6 *Application of the developed formulations*:

Both bacterial and fungal formulations were applied onto the rhizosphere in the form of spray and solid form respectively at specified intervals of time. Four sets of plants were taken into consideration with four replicates of each set. In the first set, the plant was grown on normal soil with no formulation applied to it. This set was considered as the control. In the 2nd set, the solid fungal formulation was applied at intervals of 4 weeks. In the 3rd set, the liquid bacterial formulation was applied at intervals of 4 weeks. In the 4th set, both bacterial followed by fungal formulations were applied at intervals of 2 weeks. A comparative study of the physiological growth parameters of the test plants was performed. Growth parameters including shoot length, number of internodes and leaf length were taken into account to check the efficacy of the formulations.

2.7 Chlorophyll Assay

The chlorophyll content of the leaves of plants grown in normal and activated soil was measured, followed by determination of the chlorophyll content of the leaves of plants grown in the three sets before and after application of the formulations. The procedure included collection of leaf samples from the respective test plants, preparation of the extract in methanol using 0.5g sample respectively, centrifugation followed by colorimetric estimation of the chlorophyll content at 652nm and 665nm respectively. Further, the total chlorophyll content was calculated by the formulae derived by Porra et.al (1989). The formulae devised by Porra et al. is as follows-

Chl a = $16.29A_{665} - 8.54A_{652}$

Chl b = $30.66A_{652} - 13.58A_{665}$

Chl (a+b) = $22.12A_{652} + 2.71A_{665}$

3 RESULT AND DISCUSSIONS:

On the basis of the characterisation test results, the isolated strains were identified as *Bacillus megaterium*, *Bacillus thuringiensis* and *Trichoderma viride* as shown in Figure1, Figure2 and Figure3 respectively. The interaction studies between the microbial strains indicated versatile interactive patterns between the microbial species. *B. megaterium* and *B. thuringiensis* were found to co-exist on NA with no indication of an antagonistic behaviour as shown in Figure 4. *T. viride* was found to inhibit the growth of both the bacterial strains (*B. megaterium* and *B. thuringiensis*) on both NA and PDA as shown in Figure 5.

Soil Characteristics: A pH of 7 was recorded for both the activated and normal soil.1g of normal and activated soil was subjected to drying in the Hot Air Oven for 6 hours. Thereafter, their weight was measured to be 0.8490g and 0.9890g respectively. It was inferred that the activated soil had a higher water retention capacity than the normal soil. An electrical conductance of the normal and activated soil was recorded to be 455.8µS and 685.7µS respectively. The microbial load was measured in CFU/ml wherein the activated soil was found to have a higher load as shown in Table 1. For the normal soil, $1.13x10^4$ CFU/ml was recorded in a dilution of 10^{-1} and 8.20×10^4 CFU/ml in 10^{-2}. For the activated soil, 4.52×10^4 CFU/ml was recorded in a dilution of 10^{-1} and 1.48×10^5 CFU/ml in 10^{-2}.

The set of control was prepared by growing the test plants in the normal and activated soil separately. The chlorophyll content of leaf sample obtained from the test plant grown in normal soil was measured to be 40.24µg/ml corresponding to a Mean Absorbance of 2.369 at 665nm and 1.529 at 652nm. Leaf sample obtained from the test plant grown in the activated soil was measured to be 58.82µg/ml corresponding to a Mean Absorbance of 2.593 at 665nm and 2.342 at 652nm.

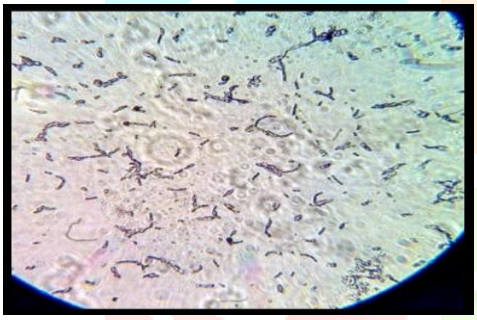

Fig.1 Gram positive rods in chains [*Bacillus thuringiensis*]

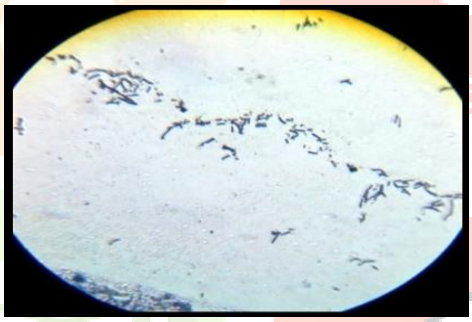

Fig.2 Gram positive rods in chains or clusters [*Bacillus megaterium*]

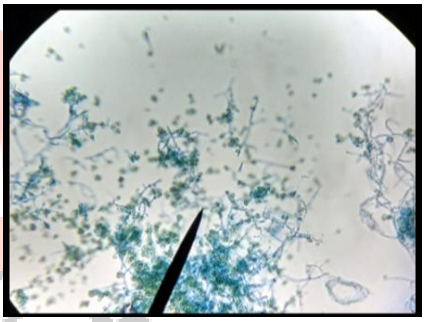

Fig.3 Septate fungal hyphae with clusters of conidia [*Trichoderma viride*]

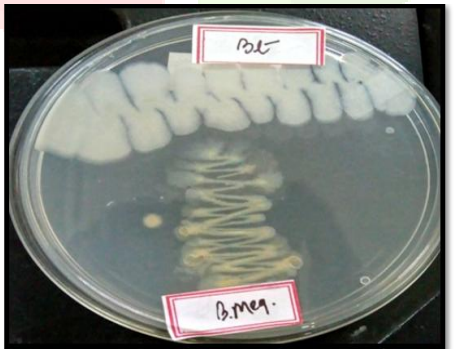

Fig.4 Interactions between *Bacillus megaterium* and *Bacillus thuringiensis* on NA

Fig.5 Interactions between *Bacillus megaterium* and *Trichoderma viride* on PDA

Table-1 Microbial load of normal and activated soil

Type of soil	Dilution	CFU/ml
1.Ground Soil	i.10^{-1}	1.13×10^{4}
	ii.10^{-2}	8.20×10^{4}
2.Activated Soil	i.10^{-1}	4.52×10^{4}
	ii.10^{-2}	1.48×10^{5}

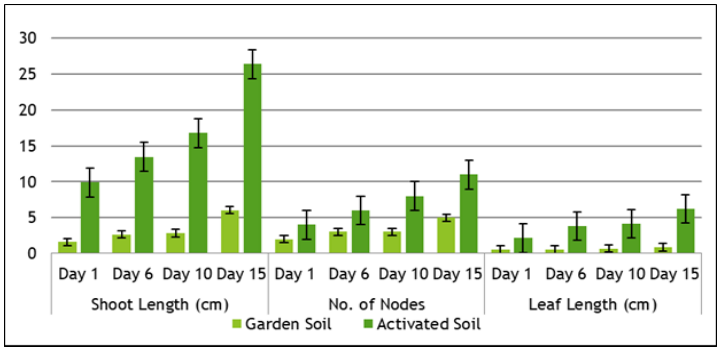

Fig.6 Comparative study of the plant growth parameters in Normal (garden) and Activated soil

Figure.6 shows the comparative study of the plant growth parameters in normal and activated soil wherein an increase in shoot length by 3.5%, increase in the number of nodes by 5.5% and an increase in the length of leaves by 1.25% were recorded in the plants grown in activated soil without the application of any formulation. The plant growth parameters were also measured for the test plants before the application of the formulations and once again after the application of the bacterial and fungal formulations. An additional set was prepared wherein both the bacterial and fungal formulations were added together and the growth parameters of the test plants were measured. The results were recorded as shown in Table 2.

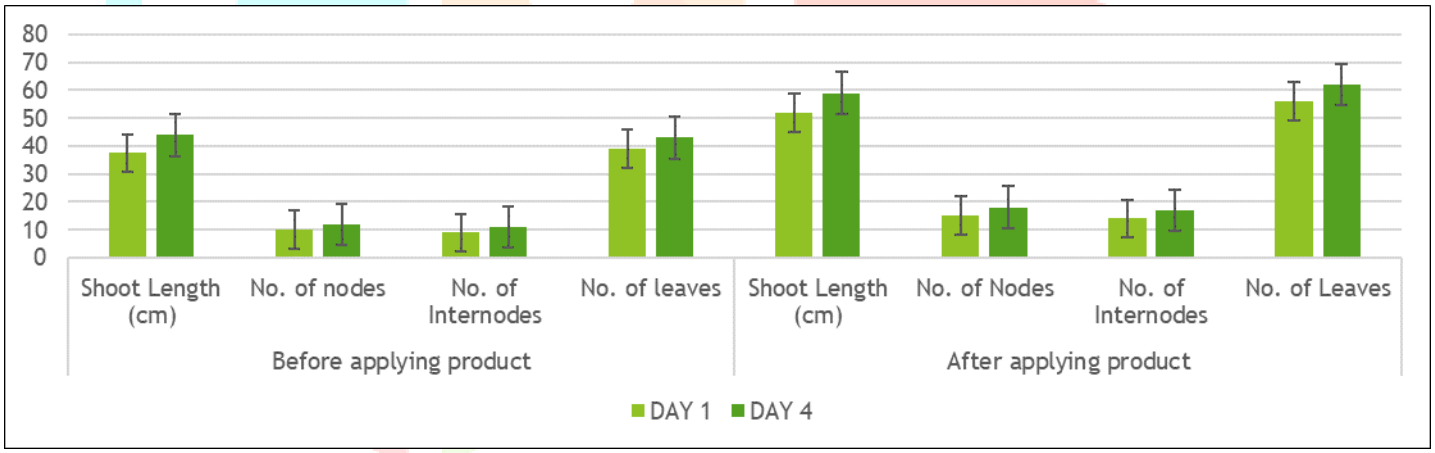

Fig.7 Comparative study of the plant growth parameters before and after application of bacterial (liquid) formulation

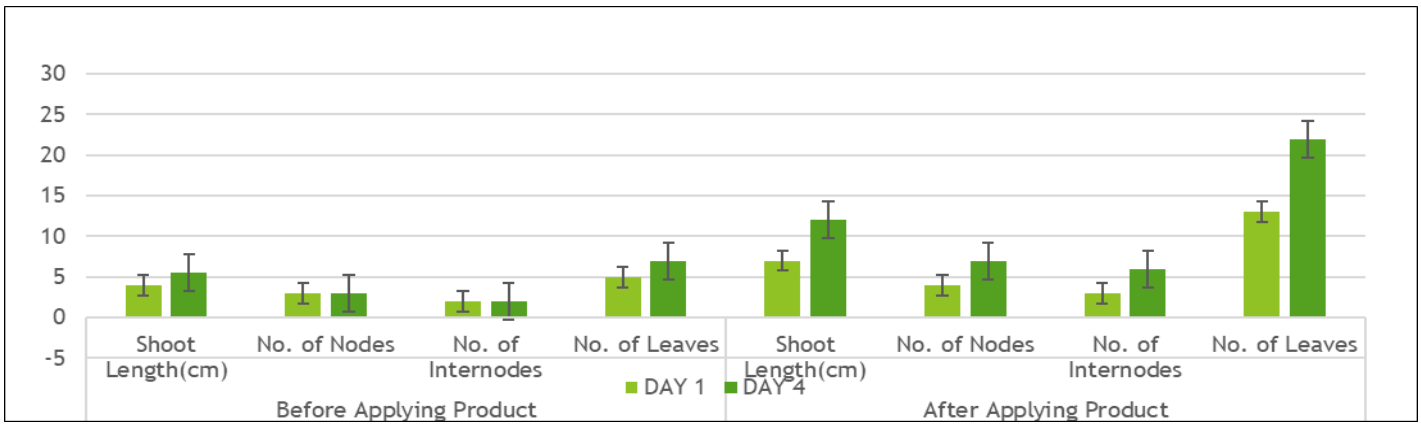

Fig.8 Comparative study of the plant growth parameters before and after application of fungal (solid) formulation

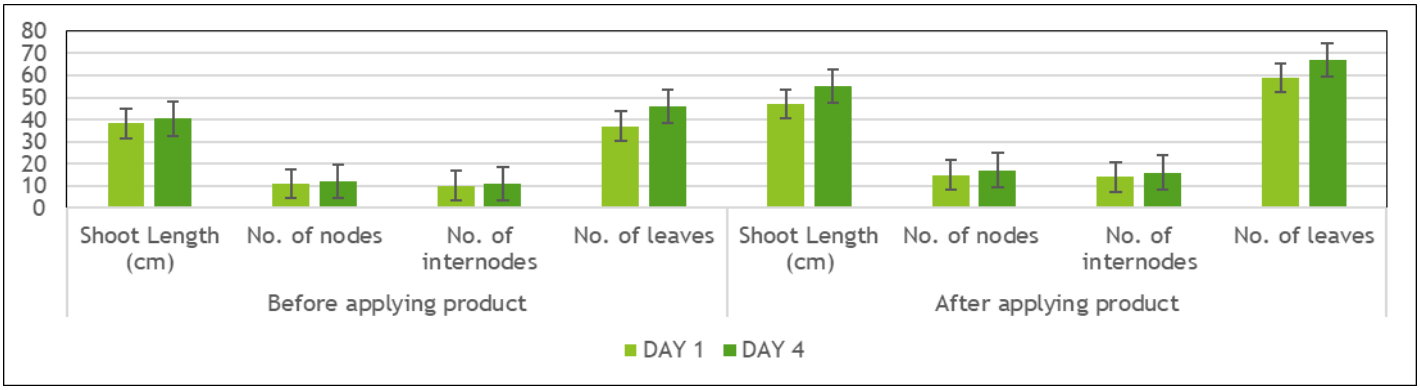

Fig.9 Comparative study of the plant growth parameters before and after application of both bacterial (liquid) and fungal (solid) formulations

Figure 7, 8 and 9 graphically represent the difference in the plant growth parameters before and after application of the formulations on the rhizosphere of test plant.

Table.2 Difference in the efficiency of the three formulations (bacterial, fungal, bacterial + fungal) as shown by % increase of the plant parameters after application on the rhizosphere of the test plant

Formulations	% increase in shoot length	% increase in number of nodes	% increase in the number of internodes	% increase in the length of leaves
Bacterial	5	2	2	7
Fungal	10	6	6	23
Bacterial + Fungal	5	1	2	8

The application of the bacterial formulation resulted in an increase in shoot length [5%], number of nodes [2%], length of internodes [2%] and the number of leaves [7%]. The application of the fungal formulation resulted in an increase in shoot length [10%], number of nodes [6%], length of internodes [6%] and the number of leaves [23%]. The bacterial and fungal formulations when applied to the same set of test plants resulted in an increase in shoot length [5%], number of nodes [1%], length of internodes [2%] and the number of leaves [8%]. A loss in efficacy of the product was observed with the co-application of both the formulations. The application of the fungal formulation resulted in a significant increase in all the plant growth parameters. The efficacy of the fungal product was hence, superior to the bacterial product.

As calculated using the formulae derived by Porra et al., the higher concentration of the total chlorophyll content in the leaves of test plant grown in activated soil as compared to the normal soil has been demonstrated graphically in Figure 10.

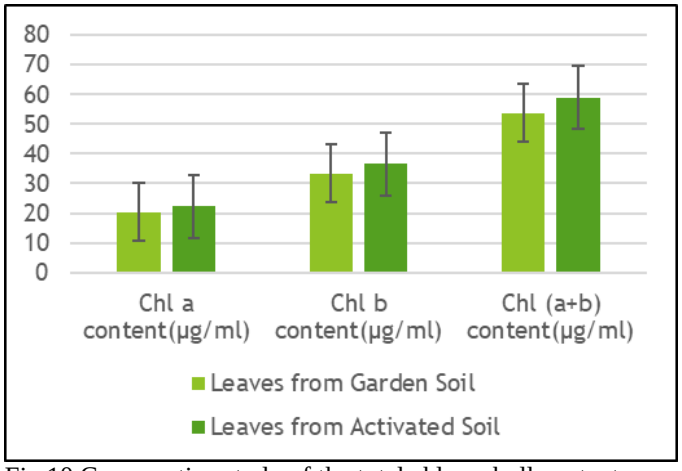

Fig.10 Comparative study of the total chlorophyll content in the leaves of test plant grown in normal and activated soil

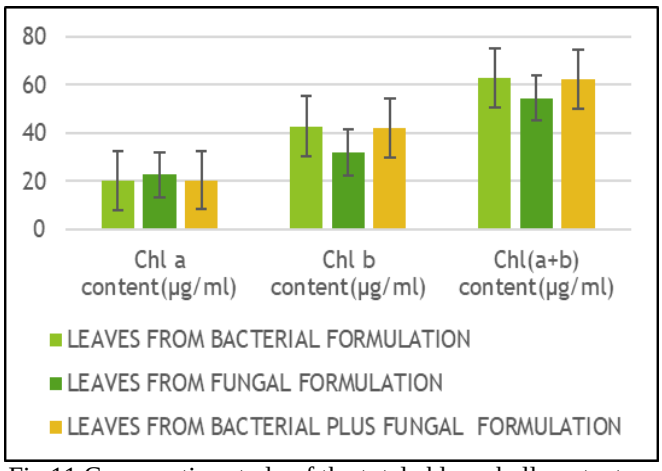

Fig.11 Comparative study of the total chlorophyll content in the leaves of test plants grown in activated soil after the application of the three formulations

Figure 11 shows the comparative study of the total chlorophyll content in the leaves of test plants grown in the activated soil after the application of the three formulations, following which it was concluded that in those sets of plant where bacterial formulation was applied had approximately equal chlorophyll content compared to those sets of plant in which both bacterial and fungal formulations were applied. In contrast, the sets of plants to which the fungal formulation was applied had considerably lesser amount of chlorophyll in their leaves, thus proving the ability of bacteria in synthesizing pigments.

Fig.12 Leaf to the left-from Normal Soil; Leaf to the right-from Activated Soil in which formulations were applied

Fig.13 Premature senescence of buds observed in the test plant grown in Normal Soil

Fig.14 Buds and flowers observed in test plants grown in activated soil with the formulations applied to it

4 CONCLUSION

The agricultural trends in the recent years have shifted primarily towards engaging in an organic approach towards cultivation of food crops. The research interest in developing bio-fertilizers, bio-pesticide, vermicompost, etc. were pioneer in this regard. Through the above conducted experiments we thrived to develop a novel plant growth promoting product using commonly available strains of *Bacillus megaterium*, *Bacillus thuringiensis* and *Trichoderma viride.* Products under a similar domain have been developed in the past. However, a major drawback of premature senescence of flower buds was observed in most of the cases. Therefore, our efforts were to combat this situation without compromising on the quality of the growth promoter. The growth factors are completely hormone based and are therefore easily diffusible into the plant. The efficacy of the cultures was found to be high as regular application of the product had given desirable results within a very short time period. Commercial feasibility was considered while preparing the formulations and therefore, it is expected to be commercially acceptable. The fungal formulation was found to be the most

effective in bringing about uniform accelerated growth of the test plant. The exact mechanism behind the inhibition of *B. megaterium* and *B. thuringiensis* by *T. viride* is not clearly understood. However, this antagonistic behaviour of the fungal member explains the reduction of efficacy when both the bacterial and fungal formulations were applied on the same set of test plants. The future of agriculture lies in that of organic cultivation of food crops and the development of biological plant growth promoting agents is an absolute necessity to replace its chemical counterparts.

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