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ORIGINAL ARTICLE

Development of Liquid Formulation of Actinobacteria and its Effect on Growth of Commercial Tea Cultivars

R. Jayanthi[1 2], P. Nepolean[2], G. Gayathri[1] and A.K. Kathireshan[1 *]
[1]Vels Institute of Science, Technology and Advanced Studies (VISTAS),
Old Pallavaram,iChennai - 600117 [2]UPASI Tea Research Institute, Valparai- 642127
Email id- *Corresponding author: kathirhodmicrobiology@gmail.com

ABSTRACT
Actinobacteria are a prominent category of gram-positive bacteria that play a significant function in the soil ecosystem, including the promotion of plant development and the production of lactic acid. Six possible actinobacteria, namely AAS2, AAS7, APSA1, APSA4, APSA5, and CAS4, were recovered from the Anamallais and Coonoor. The purpose of this study was to standardise different carrier materials, such as soil extract, rice porridge, and a vermiwash-based carrier material (0.1 percent NaCl and 0.1 percent casein), for the mass multiplication of actinobacteria strains, as well as to develop a liquid biofertilizer for use in nursery plants. The nursery experiment consisted of eight treatments, three replications, and 50 nursery plants per plot in each of the three replications. The nursery soluble mixture (NSM) was used as per recommended practice. Biometric growth parameters along with untreated controls per plant were measured on tea plants (Camellia sinensis) to determine whether the liquid formulation had growth-promoting activity. Untreated controls were used to compare the results of the experiment. According to the findings of this research, the vermiwash-based liquid carrier formulation promoted the proliferation of actinobacteria and was also beneficial in encouraging the growth of the tea plants. As an added bonus, when applied at a rate of 5 ml per plant, the formulation, which included the strains APSA1 and APSA4, produced considerably higher values of biometric growth parameters when compared to other strains & untreated controls.

Keywords: Actinobacteria, bioformulations, growth promoting activities, tea plants.

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INTRODUCTION

Actinobacteria are one of the biggest bacterial groups and are found in both aquatic and terrestrial settings. They are characterised by a high G+C DNA concentration and filamentous growth. Actinobacteria flourish in a wide variety of ecological environments due to their physiological adaptability [1]. This group of bacteria, particularly *Streptomyces*, is well-known for its ability to create a diverse array of natural products with enormous structural and biological diversity, many of which have uses in biotechnology, medicine, & agriculture [2]. Faced with global health challenges such as growing illness rates & widespread antibiotic resistance, continual appeals for novel antibiotics, chemotherapeutic medicines, and agrochemicals are made. Yet, attempts to screen soil actinobacteria for novel bioactive metabolites for therapeutic application have met with poor success in recent decades, with recurrent isolation of known compounds now being a serious challenge [3]. As a consequence, bioprospecting actinobacteria from hitherto uncharted areas like marine sediments, hydrothermal vents, arid soils, plants, & insects has been advocated as a critical technique for replenishing the medication pipeline [4].Tea is the world's second most consumed non-alcoholic drink, after water, with three billion kg manufactured and consumed annually [5]. Consumption of tea beverages is connected with a variety of therapeutic and nutritional advantages, due to the fact that tea plants (*Camellia sinensis*) produce an arsenal of nutraceuticals [6].Tea (*Camellia sinensis* (L.) O. Kuntze.), is a valuable perennial agricultural plant that is susceptible to assault by a variety of pests and diseases. Tea is one of the most popular and costly beverages worldwide due to the distinctive taste and flavour obtained from the shoots of commercially grown tea plants (*Camellia sinensis (L.) O. Kuntze*). Among the numerous crops, tea needs a

comparatively large quantity of nutrients such as nitrogen, phosphorus, and potassium. Constant fertiliser intake in order to increase yields results in nutrient depletion in the soil. Agrochemicals are often used to treat or prevent diseases, pests, and weeds in order to preserve the quality of agricultural goods and minimise production loss. While this industrialized technique reduces the cost of producing tea and therefore increases farmer earnings, substantial concerns have been made regarding health dangers, with strong dosages of chemical fertiliser, albeit developmental resistance and environmental pollution, being underlined. To ensure sustainable agriculture, it is critical to reduce the usage of these toxic chemicals and replace them with more environmentally friendly alternatives. Actinobacteria that promote plant growth development are also known as biofertilizers and biocontrol agents, since they are an environmentally benign and superior alternative to harmful fertilizers and fungicides. Actinobacteria have a critical function in encouraging plant development and antifungal activity.Actinobacteria are abundant and widespread in tea soil. They are capable of controlling tea infections and enhancing plant development primarily by solubilizing minerals by the release of organic acids & PR enzymes. Their biocontrol potential was previously investigated *in vitro*. Bioformulation as solid formulations has significant drawbacks in the field, including a shorter shelf life, increased contamination, and poor field performance. This condition may be avoided by formulation enhancements, and liquid formulation technology offers a potential alternative to the problems associated with traditional solid bioformulations. At the moment, bioformulations are mostly formed in solid carriers such as talc, lignite, and coir pith. When talc formulations are used to address tea illnesses by spraying methods, concerns have been raised about nozzle obstruction and bioinoculant dispersion.At the moment, bioformulations are mostly formed in solid carriers such as talc, lignite, and coirpith.The purpose of this research was to discover a suitable liquid carrier medium for mass propagation of actinobacteria strains as well as to investigate their potential growth enhancement impact on nursery tea plants.

MATERIAL AND METHODS

ISOLATION OF ACTINOBACTERIA

SCNA mediumwas used to isolate actinobacteria from soil (Starch 10.0g, casein 0.3g, KNO3 2.0g, NaCl2 2.0g, K2HPO4 2.0g, MgSO4.7H2 O 0.01g, Agar 20g and distilled water 1000 ml). To minimise bacterial and fungal contamination, the pH of the medium was changed to 7.2 and nystatin and nalidixic acid were added. Serial dilutions of soil samples from 10^{-4} to 10^{-7} were performed. 1ml of each aliquot was transferred to sterilised Petri plates. Each Petri plate was loaded with about 15ml of the cooled starch casein medium (45 °C) and gently rotated to mix the inoculum. The plates were incubated at 37 °C for 3–4 days (to see aerial mycelium), 7–14 days (to observe mature aerial mycelium), and 30 days (to observe mature aerial mycelium) (for slow growing isolates). The isolates were sub-cultured many times onto starch casein nitrate agar medium until single colonies formed.

MORPHOLOGICAL AND CULTURAL CHARACTERIZATION

We investigated the morphological and cultural properties of selected actinobacteria strains by inoculating them into sterile starch casein medium. Sterilized media were put onto sterile Petri dishes. After solidification of the media, the chosen strain's culture was streaked aseptically onto the medium surface and cultured at 27 °C for 7 days. A single colony was picked from each isolate and streaked on to a sterile slide coated with SCNAand incubated at 37°C for 48 hours. Two drops of methylene blue dye were then applied and left to stand for one minute. After covering the slide with a cover slip, their morphology was studied under a microscope [7]. We noticed morphological aspects such as colony characteristics and the kind of aerial hyphae.

MOLECULAR IDENTIFICATION OF ACTINOBACTERIA

Identification of actinobacteria through 16S rRNA genotyping [8] described the isolation of genomic DNA as follows: Genomic DNA was isolated from overnight cultures of all five actinobacteria antagonists using the QIAGEN DNA extraction kit (Qiagen, Valencia, CA), suspended in 100 l of elution buffer (10 mM/L Tris-HCl, pH 8.5), and quantified by measuring the optical density at 260 nm. The PCR reaction mixture included 100 ng of template DNA, 20 mol of 16S rRNA primers, 200 M dNTPs, 1.5 mM MgCl2, 1U Taq DNA polymerase, and 2 L of 10x Taq polymerase buffer. Amplification was performed using a thermocycler with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 1 minute, and final extension at 72°C for 5 min. PCR results were tested on a 1% agarose gel at 100 V for 16S rRNA amplicons.

SEQUENCE ANALYSIS OF PCR PRODUCTS

Purification of the 16S rRNA amplified fragments from the agarose gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and ligation into the pGEM®-T Easy vector (Promega Corporation, Madison, USA) were performed according to the manufacturer's instructions. Following this, the recombinant DNA

was transformed into E. coli strain DH5á and plated on Luria Bertaini agar medium supplemented with ampicillin (50 g/mL), X-Gal (20 g/mL), and IPTG (isopropyl—Dthiogalactopyranoside; 0.1 mM/L) [8]. The presence of insert DNA encoding 16S rRNA was verified in recombinants utilising PCR amplification and sequencing using an automated DNA sequencer (Model 3100, Applied Biosystems, USA). The sequences were analysed and uploaded to GenBank using the Basic Local Alignment Search Tool (BLAST) programme (http://www.ncbi.nlm.nih.gov/blast).

CARRIER OPTIMIZATION

Three different types of carriers were tested for the mass multiplication of PGPR namely 1. Rice porridge (RP) 2. Soil extract (SE) and 3. Vermiwash liquid (0.1% casein and 0.1% Nacl)

PREPARATION OF ACTINOBACTERIA SUSPENSION

Actinobacteria strains were inoculated to the 500 ml of SCNA broth individually and kept for incubation in orbital shaker at room temperature for 7 days. The 7 days old culture was concentrated and utilized for preparation of formulations.

INOCULA FORMULATION

Formulations were prepared by mixing 10 ml ofActinobacteria suspensions with carrier materials autoclaved at 121°C for 15 minutes. Inoculated formulations were kept in incubation for 7 days.The bio-formulations were stored in screw cap bottles kept under room temperature. Actinobacteria suspensions in sterile distilled water alone served as a control and all the treatments were done in three replicates.

VIABILITY ASSESSMENT

Shelf life of such bio-formulations were monitored at monthly intervals up to three months and expressed as colony forming units (cfu)/ml using dilution plate technique (Table 2 & Fig. 1). The cfu counts were recorded on seventh day after plating. cfu were estimated at regular intervals for a period of three months and the data was recorded. Based on colony population vermiwash based liquid material was selected as the best carrier material out of the tested.Each strain again inoculated in selected vermiwash based carrier material and incubated until 90 days and cell viability tests were carried out(Table.3). This vermiwash- based liquid carrier material was used for nursery experiment.

NURSERY TREATMENT DETAILS

A nursery experiment was conducted using the high yielding cultivar UPASI- 9 of Tea Research Institute (TRI) to investigate the performance of the bio-formulation. The nursery experiment had eight treatments, three replications and each plot with 50 nursery plants. The nursery soluble mixtures were applied in monthly interval and followed by the application of bio-formulations at 10 days interval. Treatment details were as follows:

T1 - *Streptomyces chartreusis* T4 - *Streptomyces xanthocidicus*
T2 - *Streptomyces flavogriseus* T5 - *Streptomyces* sp.
T3 - *Streptomyces crystallinus*T6 - *Streptomyces albus*
T7- Recommended practice T8- Control (Untreated)

RESULTS

ISOLATION OF ACTINOBACTERIA STRAINS FROM SOIL

About 30actinobacteria isolates were isolated from the soil samples collected from Anamallais by serial dilution technique. The isolates were further sub-cultured and streak plated until a single colony of each is obtained.

IDENTIFICATION OF ACTINOBACTERIA

Single colonies of the isolates were visualized under a microscope to determine the strains based on the colony morphology. By examining the colony morphology of the isolates, it was determined that they were actinobacteria.

MOLECULAR IDENTIFICATION OF ACTINOBACTERIA

Sanger sequencing was done and the sequences were analysed using BLAST which revealed that all the six potential isolates viz., AAS2, AAS7, APSA1, APSA4, APSA5 and CAS4 as actinobacteria (Table 1).

OPTIMIZATION OF CARRIER MATERIAL BASED ON ACTINOBACTERIAL POPULATION

Three different carrier materials were used namely: VW- vermiwash, SE – soil extract, RP- rice porridge. The population of actinobacteria were screened for till 90th day at different concentrations of the carrier material (Fig 1; Fig 2). Results showed that Vermiwash based liquid carrier formulation supported the growth of actinobacteria more than the other two liquid carriers, i.e., Soil extract and Rice Porridge based liquid formulation.

DEVELOPMENT OF LIQUID FORMULATION FOR FIELD APPLICATION OF ACTINOBACTERIA

The purpose of this research was to discover a suitable carrier medium for actinobacteria strains mass multiplication. Seven-day-old cultures of four established actinobacteria strains were individually injected with vermiwash and incubated for 90 days. According to the research, cell viability tests were performed utilising the liquid bio-formulation on a periodic basis using the plate count technique. The results suggested that the vermiwash-based liquid carrier formulation enhanced actinobacteria development and was also beneficial in stimulating tea plant growth. (Table 2).

EVALUATION OF ACTINOBACTERIA ON THE GROWTH OF TEA NURSERY PLANTS

The purpose of this nursery experiment was to determine the effect of various actinobacteria, including *Streptomyces chartreusis, Streptomyces flavogriseus, Streptomyces crystallinus, Streptomyces xanthocidicus, Streptomyces albus*, and *Streptomyces sp.,* on the growth of Vegetatively propagated (VP) nursery plants using the popular clone UPASI-9. The nursery experiment included eight treatments, three replications, and 50 nursery plants in each plot. In the conventional therapy, nursery soluble mixtures (NSM) were administered according to the indicated timetable. Actinobacteria formulations were administered at a rate of 5 ml per plant. Nursery soluble mixture was administered monthly, followed by actinobacteria at 10-days intervals at the above-mentioned doses. The study's findings established that plants treated using *Streptomyces crystallinus*(APSA1) accompanied by *Streptomyces xanthocidicus*(APSA4) at a concentration of 5 ml per plant had dramatically improved biometric parameters.

TABLE 1: Molecular identification of actinobacteria

S.No.	Isolates	*Species*	*NCBI accession No.*
1	AAS2	*Streptomyces chartreusis*	*KP00444*
2	AAS7	*Streptomyces flavogriseus*	*KM 06711*
3	APSA1	*Streptomyces crystallinus*	*KM067119*
4	APSA4	*Streptomyces xanthocidicus*	*KM067120*
5	APSA 5	*Streptomyces sp*	*IMI No. 504703*
6	CAS4	*Streptomyces albus*	*KM067121*

TABLE 2: Shelf Life of Actinobacteria in Vermiwash Based Carrier Material

	Days of Incubation					
Strains	**7th**	**15th**	**45th**	**60th**	**75th**	**90th**
Streptomyces flavogriseus	Numerous	15.2	12.8	12.0	10.7	7.2
Streptomyces crystallinus	Numerous	18.2	15.2	10.7	5.6	3.8
Streptomyces albus	Numerous	Numerous	19.2	19.2	15.2	8.4
Streptomyces sp.	Numerous	15.0	14.2	13.0	7.8	2.0
Streptomyces flavogriseus	Numerous	17.0	15.0	9.0	7.0	3.0
Streptomyces xanthocidicus	Numerous	20.0	16.2	11.0	9.0	7.0

Values indicate colony forming units ($x10^9$) per ml.

TABLE 3. Evaluation of bio-formulations on the growth of nursery plant in tea (ten months)

Treatment	Leaf count (No.)	Collar Diameter (mm)	Shoot length (cm)	Root length (cm)	Shoot dry weight (g)	Root dry weight (g)	No. of lateral root
Streptomyces chartreusis	16.3	2.5	49.6	32.5	4.2	3.8	2.5
Streptomyces flavogriseus	18.5	2.8	44.2	36.1	3.6	2.8	2.8
Streptomyces crystallinus	23.2	3.1	55.1	34.3	6.2	6.0	3.1
Streptomyces xanthocidicus	20.8	3.1	50.0	31.7	6.6	5.9	3.1
Streptomyces sp.	20.0	3.3	56.1	34.5	6.8	8.0	3.3
Streptomyces albus	18.8	2.5	42.8	33.3	4.1	3.9	2.5
Recommended practice	12.8	2.4	49.8	30.0	4.0	2.9	2.4
Control (Untreated)	13.8	2.4	33.0	27.1	3.8	4.4	2.4
CD @0.05%	9.2	0.6	4.4	7.8	0.4	0.7	0.6

FIGURE 1: Growth of actinobacteria at different concentration of different Liquid Carrier Formulation. (VW- VERMIWASH BASED; SE- SOIL EXTRACT; RP- RICE PORIDGE)

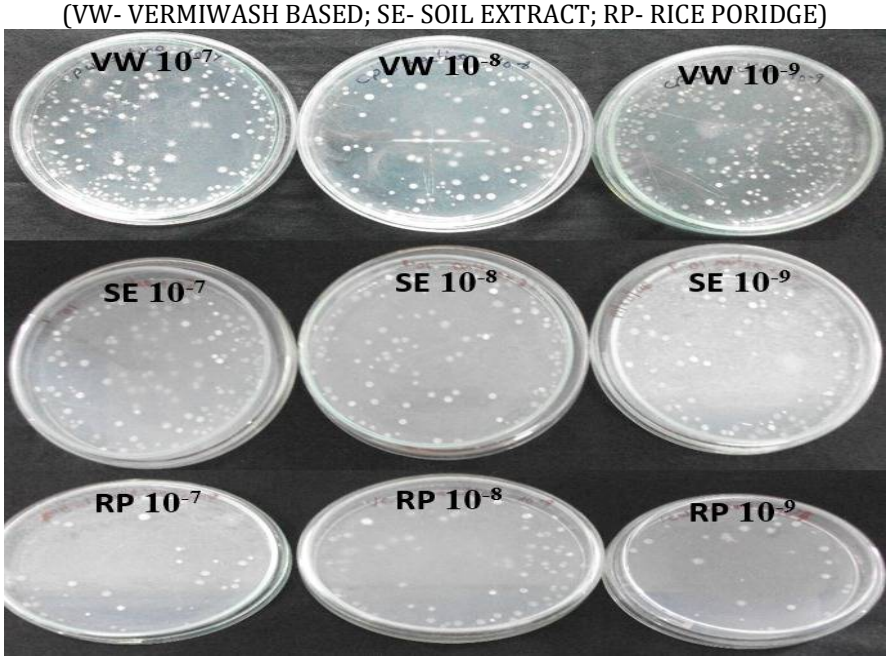
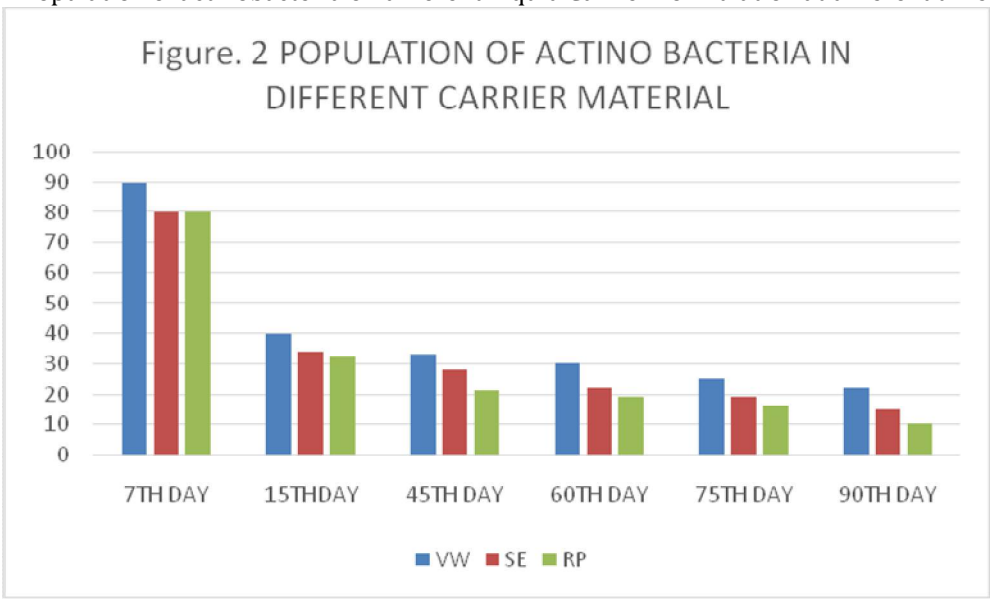


FIGURE 2: Population of actinobacteria on different Liquid Carrier Formulation at different time interval.



VW – Vermiwash, SE – Soil Extract, RP – Rice Porridge

DISCUSSION

Bio manures are generally prepared as carrier-based inoculants encompassing actual microbes. Assimilation of microbes in carrier materials promotes long shelf life, efficiency and eases handling of bio fertilizers. Basically, various carrier materials are being used such as lignite, vermiculite, charcoal, agro industrial waste, compost etc. The competence of several types of bio inoculants is diverse depending on the nature of the carrier material used. Hence, choice of carrier material is a crucial step in the bio fertilizer production and in the crop response as well [9]. Quality of bio-formulations is a key factor defining their performance. An ideal carrier material should keep up the viability of microbes for a longer period while holding the moisture content [10].Currently, bio-formulations are created mostly in solid carriers such as talc, lignite, and coir pith. As the available solid formulations, field and nursery applications of bio-formulations suffer from significant drawbacks such as shorter shelf life, higher levels of contamination, and poor field performance. This condition could be avoided with formulation advancements, and the liquid formulation technology is a potential solution for overcoming the shortcomings of traditional solid bio-formulations, which are now being investigated. On the other hand, the microbes that are being used for preparation of bio-formulations are the key players in mobilization

of minerals to the plants thereby improving the growth and development of plants[11]. The present study was to formulate a suitable liquid carrier material for the mass multiplication of actinobacteria strains, as well as to investigate their potential influence on plant growth promotion in nursery tea plants.Based on the findings, it has been established that, of the three liquid-based carrier materials tested, the vermiwash-based liquid carrier formulation was the most successful in stimulating the development of actinobacteria and the most effective in boosting the activity of the tea plants. Improvement in shoot length, root length, shoot dry weight, root dry weight, leaf count, collar diameter and number of lateral roots was prominent by inoculation of Actinobacteria strains in combination with vermiwash liquid.

CONCLUSION

Thus, we would like to conclude that this vermiwash-based liquid formulation with Actinobacteriaare environmentally safe, and they could be considered as suitable strains for use as bioformulations in the promotion of the development of tea plants.

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