Development of Liquid Formulation of native *Rhizobium sp.* for effective plant nourishment

Minal Trivedi[1*], Arti Shanware[2], Surekha Kalkar[3]

[*1]Research Student, Rajiv Gandhi Biotechnology Centre, Laxminarayan Institute of Technology Campus, Rashtrasant Tukadoji Maharaj Nagpur University, Amravati Road, Nagpur, Maharashtra, India
[2]Assistant Professor, Rajiv Gandhi Biotechnology Centre, Laxminarayan Institute of Technology Campus, Rashtrasant Tukadoji Maharaj Nagpur University, Amravati Road, Nagpur, Maharashtra, India
[3]Associate Professor, Department of Botany, Govt. Institute of Science, Nagpur-440001(M.S.), India

Abstract— *In the current study bacteria having potential to fix nitrogen symbiotically were isolated from Soybean plant roots on Yeast extract Mannitol agar from roots. They were screened on basis of Acetylene gas reduction assay & various plant growth promoting factors. They were subjected to morphological, biochemical & molecular characterization. The liquid formulations were made by amendments with additives & emulsifier in the liquid carrier of most potential strain SNFB 03 was identified as Rhizobium sp. It was found to be most effective on the Soybean growth parameters under green house studies.*

Keywords— Additives, Liquid formulation, Rhizobium sp., Soybean, Symbiotic Nitrogen fixer.

I. INTRODUCTION

Daniel Rutherford in 1772 discovered Nitrogen which is colorless & odorless .There is a triple bond present in the di Nitrogen (N_2) which readily can't accept or donate electrons. Nitrogen fixation is a key process in which molecular Nitrogen is reduced to form ammonia, the form of Nitrogen that is used by living systems for the synthesis of many bio-organic compounds. The Nitrogen cycle can be defined as transfer among the inorganic forms of Nitrogen. Few microorganisms have ability to fix atmospheric Nitrogen, so as to make it available to plants in usable form. With increase in world population, there has been increased reliability on Nitrogen fertilizers to meet with the demands of food and economic growth rates. This reliability on synthetic chemicals causes environmental problems. Nowadays we are exploiting Biological Nitrogen fixation process for an eco friendly approach for sustainable agriculture. Biological Nitrogen-fixation is a complex process that involves a number of functional and regulatory gene products [1]

The Nitrogen in the basic component of Nucleic acids (i.e. both DNA & RNA) and Proteins. It is required for the production of Chlorophyll-the plant pigment .Deficiency in Nitrogen is detected by necrosis of older leaves, slow and stunted growth, chlorosis of lower leaves, Yellow coloration from leaf tip. The yield is reduced in case of Nitrogen deficient crops as they mature early, bear few tillers, grain yield is low in protein content [2]. For past many decades biofertilizers are being used to replace chemical fertilizers. Currently used biofertilizer are solid carrier based which have drawback of low shelf life, unable to sustain adverse condition, upon application they form clumps leading to reduced efficiency. To overcome the drawbacks of the solid based biofertilizer, liquid bioinoculant have been developed. Liquid biofertilizers are unique liquid formulation containing the desired beneficial microorganisms and their biological secretions, along with special cell protectants or substances that encourage the formation of dormant spores or cysts for longer shelf life and tolerance to adverse conditions [3].This study aimed at isolation and formulation of liquid bioinoculant of competent symbiotic Nitrogen fixers from Soyabean plant rhizosphere. Green house studies were conducted to check the efficacy of competent liquid bioinoculant on the Soybean plant.

II. MATERIAL AND METHOD

2.1 Procurement of Standard strain

The Standard strain *Rhizobium leguminosarum (*MTCC 9766) was procured from the Institute of Microbial Technology (IMTECH), Chandigarh & was maintained on Yeast extract mannitol agar.

2.2 Isolation of Symbiotic Nitrogen fixers

Symbiotically Nitrogen fixing bacteria were isolated from the root samples collected from different sites of Soybean fields located at Kuhi, Hingna, Dahegaon (Nagpur, Maharashtra).The roots were surface sterilized by 70% ethanol for 30 s followed by treatment with 2% sodium hypochlorite for 5 min and then washed with sterilized distilled water, twice . The

sterilized roots were aseptically cut into 1–2 cm sections, macerated with 0.8% saline solution and then decimally diluted in 0.8% saline solution. The Higher dilutions were plated on Yeast extract mannitol agar medium containing Congo Red as indicator (CRYEMA) and incubated at 30 ± 2 ºC for 48-36 hrs [4].

2.3 Screening based on Acetylene gas reduction assay

Nitrogen fixation capacity of the isolates obtained on Jensen's medium & Yeast extract mannitol medium containing Congo red were determined with the help of Acetylene reduction assay (ARA) [5]. Nitrogen fixing bacteria were screened indirectly i.e.by measuring the reduction of acetylene to ethylene.

2.4 In vitro assessment of plant growth promoting substance of the test isolates

2.4.1 Production of Indole Acetic Acid

The potential bacterial cultures were screened for their ability of converting L-Tryptophan into Indole acetic acid compounds. The estimation of IAA produced was performed by a colorimetric method that used Salkowski reagent containing ferric chloride and perchloric acid [6]

2.4.2 Production of siderophore

The test bacteria were screened for the siderophore production by adapting the universal chemical assay (Chrome Azurol S assay) as explained by Schwyn and Neilands [7].

2.4.3 Production of ammonia

Detection of ammonia production by isolated microorganisms qualitatively was done as described by Bakker and Schippers [8] that used Nessler's reagent.

2.5 Morphological, Biochemical characterization

The native Nitrogen fixing bacteria were identified morphologically on the basis of Gram staining, motility and biochemical characteristics according to the standard methods described in Bergey's manual of Systematic Bacteriology [9] and Laboratory Manual of Basic Microbiology [10].

2.6 Molecular characterization

To confirm the identity of the biofertilizer strain genomic DNA was extracted as described by Marmur (1961). 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) with the help of universal primers for bacteria .DNA sequencing reaction of PCR amplicon was carried out. The 16S rRNA gene sequence was subjected to BLAST analysis. The sequences were aligned using multiple alignment software program Clustal W. The phylogenetic tree was constructed using MEGA 4 [11].

2.7 Preparation of Liquid Bioinoculant & shelf life studies

The liquid bioinoculant was prepared by various combinations of Polyvinyl pyrrolidone (PVP) (at 1%, 2.5%, 5%) & Polysorbate 60 (0.05%) in the liquid carrier that was Nutrient broth amended with glycerol (2%)(NBG) .The test bacterial culture was added at rate of 1% in each batch. They were incubated at 28 ± 2^{o}C for 48-72 h under shaking at 150 rev/ min. The fermented broth was packed in the High-density polyethylene (HDPE) bottles .The survival of strain(s) was enumerated at monthly intervals up to decline phase by serial dilution technique.

2.8 Green House studies

In order to see the primary effects of the developed liquid bioinoculant on the growth of different plants, the Green house experiments were carried out at Rajiv Gandhi Biotechnology Centre, Nagpur, Maharashtra.Seeds were surface-sterilized in ethyl alcohol (70%) for 1 min, immersed in HgCl2 (1:500) for 0.5 min, and washed 6 times with sterile water. The seeds were primed with the chemical fertilizer & the biofertilizer as per the treatment required (Table 1). Plants were grown in a greenhouse under natural light supplemented with a daily 12h: 12h light: dark photoperiod. Greenhouse temperatures were maintained at 25°C (days) and 14°C (nights) with 20–50% relative humidity. The experiment was set up in triplicates in completely randomized design.

TABLE 1
GREEN HOUSE TREATMENTS FOR SOYBEAN PLANT

S.№	Code	Treatments
1	A	Control
2	A_1	Chemical fertilizer (Urea)
3	A_2	Commercially available Solid biofertilizer of *Rhizobium*
4	A_3	Developed liquid bioinoculant of *Rhizobium sp.*

Various plant parameters like Plant Height (cm), Root Length (cm), no. of branches/plant, no. of pods/plant were measured manually taking mean of three replicates. The Chlorophyll content (mg/gfw) of the leaves was established following the method proposed by Anderson and Boardman [12] .Rhizobium population (log CFU/mL) from each soil sample with respective treatments by serial dilution on CRYEMA [4].

2.9 Statistical analysis

The data were subjected to analysis of variance. The differences among various treatment means were compared using the least significant differences test (LSD) at 5% ($P \leq 0.05$) probability level using Windows at package, Version 8.0 [13].

III. RESULTS & DISCUSSION

3.1 Isolation & Screening of Symbiotic Nitrogen fixers

A total of 15 soil samples were collected and 36 isolates were obtained on CRYEMA. These were screened on basis of acetylene reduction assay,18 isolates showed more than 150 nmole C_2H_4 $h^{-1}mg^{-1}$.This same criteria was used by Park *et.al.* [14] for screening Nitrogen fixers. Based on Acetylene gas reduction assay the strains SNFB 03 (440.58 nmole C_2H_4 $h^{-1}mg^{-1}$), SNFB 04 (398.58 nmole C_2H_4 $h^{-1}mg^{-1}$), SNFB 09 (414.33 nmole C_2H_4 $h^{-1}mg^{-1}$) showed higher Nitrogenase activity compared to standard strain *R.leguminosarum* (394.48 nmole C_2H_4 $h^{-1}mg^{-1}$).The range of Nitrogenase activity in symbiotic Nitrogen fixers was 151.40 nmole C_2H_4 $h^{-1}mg^{-1}$ - 440.58 nmole C_2H_4 $h^{-1}mg^{-1}$ .The data was found to be significant (Table 2)

TABLE 2
NITROGENASE ACTIVITY OF NATIVE ISOLATES FROM SOYBEAN CROP RHIZOSPHERE

S.№	Isolate (SNFB)	Nitrogenase activity (nmole C_2H_4 $h^{-1}mg^{-1}$)
1	SNFB 01	219.72h
2	SNFB 02	194.37k
3	SNFB 03	440.58a
4	SNFB04	398.58c
5	SNFB05	201.50j
6	SNFB06	312.12f
7	SNFB07	151.40q
8	SNFB08	346.63e
9	SNFB09	414.33b
10	SNFB10	182.23l
11	SNFB11	159.49n
12	SNFB12	170.79m
13	SNFB13	258.57g
14	SNFB14	157.23op
15	SNFB15	204.40i
R.leguminosarum		**394.48d**
C.D. (at 5%)		**0.074**
C.V.		**0.017**

Each value represents mean of three replicates .in the same column, significant differences according to LSD at P≤0.01 levels are indicated by different letters. Same letters represent that their values are at par.

3.2 Evaluation of Biofertilizer isolates for plant growth promoting traits

The symbiotic Nitrogen fixers were further evaluated for plant growth promoting traits like IAA, siderophore & ammonia production. For assessment of IAA production, in this study L-tryptophan was supplemented in the culture medium which agrees with the work done by various workers that, presence of L-Tryptophan is essential in culture medium for boosting IAA production by the microorganisms [15,16,17]. The presence of siderophore producing microorganisms is essential for plants to utilize Fe or else all the plants would become Iron deficient. Another plant growth promoting substance is ammonia which is produced by various soil microbes. This is done by hydrolyzing 1-aminocyclopropane-1 carboxylic acid (ACC) the precursor of ethylene which affects number of biological activities in plants growth and development like promoting root initiation, fruit ripening etc., ACC deaminase catalyses this reaction hydrolyzing ACC to α-ketobutyrate and ammonia [18]. The plant growth promoting traits are summarized below in Table 3.

TABLE 3
SUMMARY OF DIFFERENT PLANT GROWTH PROMOTING TRAITS SHOW BY SYMBIOTIC NITROGEN FIXERS

S.№	Isolate (Nitrogen Fixers)	IAA concentration (µg/mL)	Siderophore production	Ammonia production*
1	SNFB01	12.26d	-	+
2	SNFB02	10.2g	-	+
3	SNFB03	14.54a	+	++
4	SNFB04	-	-	-
5	SNFB05	13.15c	-	+
6	SNFB06	14.4a	+	++
7	SNFB07	-	-	-
8	SNFB08	8.88i	+	+
9	SNFB09	11.81e	+	+
10	SNFB10	13.52b	-	-
11	SNFB11	9.81h	+	++
12	SNFB12	10.56	+	+
13	SNFB13	7.3k	-	++
14	SNFB14	-	-	+
15	SNFB15	8.9j	-	++
R.leguminosarum		**14.36a**	**+**	++
C.D. (at 5%) = 0.196				
C.V. = 1.258				

Each value represents mean of three replicates .in the same column, significant differences according to LSD at P≤0.05 levels are indicated by different letters. Same letters represent that their values are at par
**** +: Faint Yellow (small amount of ammonia) ++: deep yellow to brownish (maximum amount of ammonia production)***

3.3 Morphological & Biochemical characterization

There are various genera in the rhizospheric soil(s) that shows microbial diversity that exists in specialized niches of different plants [19]. Among the strains isolated from Soybean plant rhizosphere most of them were found to belong to genus *Rhizobium* supporting the presence of *Rhizobium* species in the root nodules (Table 4).

TABLE 4
MORPHOLOGICAL & BIOCHEMICAL CHARACTERIZATION OF NATIVE ISOLATES.

Isolae	Morphology	Gram staining	Motility	I	MR	VP	Ci	O	C	H_2S	Starch	Nitrate	U	Utilization of Sugar					Possible genus
														D	L	M	R	S	
SNFB 01	Rods	-	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	+	*Enterobacter*
SNFB 02	Rods	-	+	-	-	-	-				+	+		-	-	+		+	*Stenotrophomonas*
SNFB 03	Rods	-	+	+	-	-	-	-	+	-	+	+	-	-	-	+	-	-	*Rhizobium*
SNFB 04	Rods	-	+	-	-	-	-	-	+	-	-	+	+	-	-	+	-	+	*Rhizobium*
SNFB 05	Rods	-	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-	*Enterobacter*
SNFB 06	Rods	-	+	-	+	-	-	-	+	+	-	+	-	-	-	+	-	-	*Azospirillum*
SNFB 07	Rods	-	+	+	-	-	-	-	+	-	-		+	-	-	+	-	-	*Klebshiella*
SNFB 08	Rods	-	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	+	*Rhizobium*
SNFB 09	Rods	-	+	-	-	-	-	-	+	-	+	+	-	-	-	+	-	-	*Rhizobium*
SNFB 10	Rods	-	+	+	-	-	-	-	+	+	-	+	+	-	-	+	-	-	*Azotobacter*
SNFB 11	Rods	-	+	+	-	-	-	-	+	-	-	+	+	-	-	+	-	-	*Klebshiella*
SNFB 12	Rods	-	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	-	*Rhizobium*
SNFB 13	Rods	-	+	-	-	-	-	-	+	-	+	-	+	-	-	+	-	-	*Rhizobium*
SNFB 14	Rods	-	+	-	-	-	-	-	+	-	+	-	+	-	-	+	-	-	*Rhizobium*
SNFB 15	Rods	-	+	-	-	+	+	+	-	-	-	+	-	-	+	+	-	-	*Pseudomonas*

I – Indole, MR- Methyl red, VP-Voges Proskauer, Ci-Citrate utilization,O-oxidase,C-catalase,u-Urease,D Dextrose,L-Lactose, M-Mannitol, R-Rhamnose,S-sucrose

3.4 Molecular Characterization

Based on above screening, SNFB 03 isolate was found to be most efficient bacteria possesing Nitrogen fixing property along with all the evaluated plant growth promoting traits. An attempt was made to characterize this efficient bacteria isolated from Soybean crop rhizosphere using 16S rRNA gene sequencing to decipher the phylogenetic affiliation of this bacteria. SNFB 03 showed 90% similarity with *Rhizobium sp.* The nucleotide sequences of this isolate were deposited in GenBAnk of NCBI as '*Rhizobium sp. SAM1*' and the accession No. so obtained was 'KU982642'.The figure 1 represents the phylogenetic tree of SNFB 03 isolate.

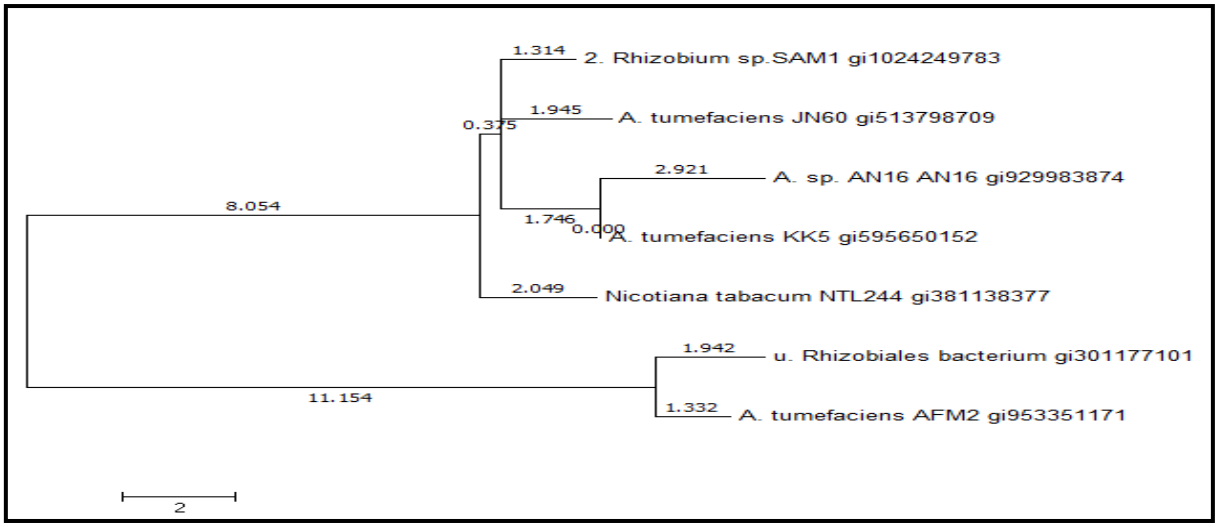

FIGURE 1.PHYLOGENETIC TREE OF SNFB 03 (*RHIZOBIUM SP.* SAM1) GENERATED USING MEGA 7.

3.5 Preparation of Liquid Bioinoculant & shelf life studies

Carrier is an important component in biofertilizer technology .It's the vehicle to carry the crux i.e. the bacterial cell, undamaged from lab to land. Among the various amendments made with additives & emulsifier in Nutrient broth (containing 2% glycerol), the PVP (2.5%) showed higher survival of bacterial cell till 720 days. PVP has a high water binding capacity, which could maintain water around the cells helping their metabolism [20] resulting in higher cell survival. The survival of *Rhizobium sp.* In other amendments on various days is represented in Table 5

TABLE 5

SURVIVAL OF *RHIZOBIUM SP.* IN DIFFERENT ADDITIVES ALONG WITH POLYSORBATE 60 IN NBG

Additives	*180th day*	*360th day*	*540th day*	*720th day*	*900th day*	*Mean*
PVP 1%	12.436	11.892	10.875	9.869	7.875	10.5894
PVP 2.5%	12.483	11.949	10.919	9.903	7.898	10.6304
PVP 5%	12.456	11.919	10.909	9.886	7.886	10.6112
Control	9.602	8.477	7.954	6.845	4.778	7.5312
Mean	11.74425	11.05925	10.16425	9.12575	7.10925	

C.V. - 0.432; C.D. (at 5%) - 0.215

3.6 Green House studies

The green house studies were conducted on Soybean for screening the effects of chemical fertilizer, solid based biofertilizer, and developed liquid bioinoculant (Figure 2)

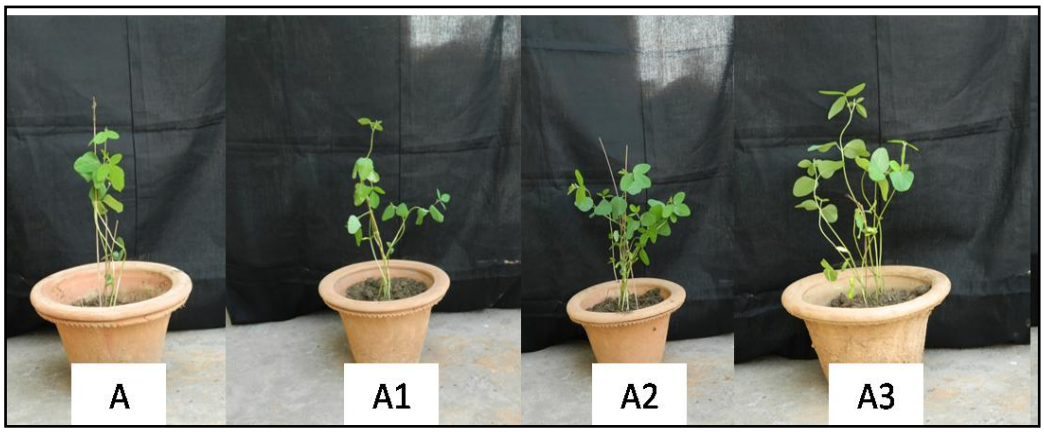

FIGURE 2. EFFECT OF DIFFERENT TREATMENTS ON SOYBEAN PLANT(S) UNDER GREEN HOUSE STUDY.

The results so obtained reflected higher values of plant height, root length, no. of branches/plant, no. of pods/plant, Chlorophyll content, Rhizobium population when compared to control, chemical fertilizer & solid based biofertilizer treatment (Table 6).

TABLE 6
EFFECT OF DIFFERENT TREATMENTS ON SOYBEAN GROWTH PARAMETERS

Treatments	A	A1	A2	A3	C.D. at 5%	C.V.
Plant Height (cm)	35.45c	41.23b	39.89d	43.35a	0.035	0.032
Root Length (cm)	8.72d	8.91c	9.52b	9.70a	0.044	0.172
No. of branches/plant	1.90d	2.26b	2.25c	2.28a	0.034	0.615
No. of pods/plant	19.30d	23.12c	27.92b	31.20a	0.042	0.163
Chlorophyll content (mg/gfw)	2.25d	2.30c	2.32b	2.35a	0.049	0.634
Rhizobium population (log CFU/mL)	7.69d	7.78c	10.07b	10.54a	0.067	0.406

Any two means not sharing a letter in common differ significantly at 5% probability level

The liquid bioinoculant of *Rhizobium sp.* showed higher values in all the parameter than the other treatments. The Rhizobium population was more in the developed liquid bioinoculant indicating nourished soil microflora. The data was found to be statistically significant, hence advocating positive effect of the liquid bioinoculant on the plant growth.

IV. CONCLUSION

In recent years much attention has been paid to natural agricultural practices in expectation of moving towards environmentally sustainable development. Current trends in agriculture are focusing on the diminution of the use of chemical pesticides and inorganic fertilizers, compelling the search for alternative ways to improve soil fertility and crop production. In current investigation a liquid formulation of symbiotic Nitrogen fixer was made and its efficacy was studied at green house level. This study advocates the application of liquid microbial inoculants as Potential biofertilizers. This liquid bioinoculant provides low-input cost in agricultural systems for decomposition of organic matter and release of nutrients for enhancing plant growth.

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