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ENHANCEMENT OF COFFEE PRODUCTION BY USING ADVANCED FORMULATION OF ZINC SOLUBILISING THIOBACILLUS sp. IN PULNEY HILLS

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ABSTRACT

Zinc is one of the eight significant micronutrients needed for the healthy growth replication of crop plants. Zinc is a micro nutrient essential for all living organism with a key role in growth, development and defence. Micronutrients role in plant defence are predominantly documented for Mn, Cu, Fe and Zn. There zinc seems to be a major player in both plant and animal immune system. Deficiency of Zinc is world wide spread problem in agricultural fields. Competition for zinc affects the outcome of the host – attacker interaction in both plant and animal system. Hence, we provide a clear framework of the Zinc solubilisation by *Thiobacillus* isolates as an immense important in Zinc nutrition to plant majorly in field having alkaline and Rhizosphere soil. The four bacterial colonies with different colony morphology were selected from different plates for Gram's staining and biochemical tests (Citrate, MR-VP, Oxidase, Catalase,) and further Plant growth promoting activity (PGPA) (zinc solubilisation). Zinc solubilisation medium contain 0.1% of zinc oxide was used and based on the solubilisation isolates are selected for zinc solubilisation efficiency for multiple plant growth promoting traits. These plant growth promoting traits can be exploited as a potential bio fertilizers.

KEYWORDS: Zinc solubilisation, Zinc oxide, *Thiobacillus sp.*, Capsule, Biofertilizer, Rhizosphere.

INTRODUCTION

Since the beginning of the Green Revolution in the last 60s, focus on crop productivity, through high yielding varieties, agrochemicals, irritation system and chemical fertilizers were made and used in large scale (Anonymous, 2008). Importance of fertilizer in yield improvement which increased agricultural production will be further more increased by bringing more area under cultivation and majority of soil are deficient in many micro and macro nutrients will be given care (Fertiliser Association of India, 2011). The significance of chemical fertilizer, sometimes led to injudicious application for soil (Rakshit *et al*., 2015). In case of these problems the use of organic fertilizers, bio fertilizers and other microbial products are essential to make the agriculture industry a viable component of a healthy and pleasant eco system.

The use of microorganisms with the aim of improving nutrients availability for plants is an important practice and necessary for agriculture (Freitas *et al*., 2007). Rhizosphere is the place where very important and intensive interactions take place between plants, soil, microorganisms and soil micro fauna, influenced by compounds exuded by roots, and microorganism feeding on its compounds (Antoun and Prevost, 2006). Microorganism are involved in the secretion of plant growth hormones, zinc, sulphur and phosphate solubilisation, protection of plant from pathogen through antibiotic production (O'Sullibian and O'Gara, 1992).

Zinc is an important micronutrient for human beings, animals as well as crops. Zn is an important component of different enzymes catalyzing many metabolic reactions in plants. It involves the processes of life in plants are influenced by Zn, such as (a) nitrogen metabolism i.e. nitrogen and protein uptake quality; (b) photosynthesis i.e. synthesis of chlorophyll and carbon anhydrase activity (c) resistance against biotic and abiotic stresses i.e. resistant against oxidative damage (Alloway, 2004). Zinc also plays a significant role in plant resistance against diseases, photosynthesis, cell membrane integrity, protein synthesis, pollen formation (Gurmani *et al.*, 2012) and enhances the level of

antioxidant enzymes and chlorophyll within plant tissues (Sbartai *et al.*, 2011).

Among the commercial crop plant, Coffee is the favorite drink and stands second place in the international commodity than to oil. Coffee is cultivated in lower Pulney hills of Dindigul district and Yercaud of Salem district in Tamil Nadu. Few Zinc solubilizing viz, *Thiobacillus thioxidans, Thiobacillus ferroxidans, Acinetobacter, Bacillus, Glucanoacetobacter, Pseudomonas* and facultative thermophilic iron oxidizers have been reported as a zinc solubilizers (Saravanan *et al*., 2007). 50% of agricultural soils contain low level of available zinc, mainly due to high soil pH, low soil moisture and low organic matter (Alloway, 2008; Cakmak 2008). Under Zn deficiency, root cell membrane permeability is increased which might be related to the function of Zn in cell membranes (Parker *et al.*, 1992). Exogenous application of zinc fertilizer has been applied to various crops and this causes transformation of about 96-99 percent of applied available zinc to various unavailable forms. This unavailable zinc can be reverted back to available form by inoculating a bacterial strain capable of zinc solubilizing potential (Saravanan *et al*., 2003). Aim of this research was to design the screening and formulation for *Thiobacillus thioxidans*, as Zinc solubilizer found in coffee rhizosphere region.

Thiobacillus thioxidan is the gram-negative rod shaped gamma proteobacteria (Kelly and Wood 2000) that metabolize sulphur as a method to obtain energy and the optimum temperature for growth is 28-30°C.

Biofertilizer and Types

Biofertilizer are environment friendly, non-bulky, low cost, renewable source of plant nutrient with supplement chemical fertilizer and play a significant role in improving nutrient supply and their crop availability in the years to come (Bisen *et al.*, 2015).

Azospirillum, Azatobacter, Bacillus, Thiobacillus, Enterobacter, Klebsiella and *Pseudomonas* are well known genera of Plant Growth Promoting Rhizobacteria (PGPR). The plant growth promotion has several mechanism which includes biological nitrogen fixation (BNF), synthesis of phytochrome, environmental stress relief, synergism with other bacterial-plant interactions, inhibition of plant ethylene synthesis as well as increasing availability of nutrients like phosphorous, zinc, sulphur, iron and minor elements and growth enhancement by volatile compounds.

The types of bio fertilizers available to the farmers in India are, Nitrogen-fixing bio fertilizers (*Rhizobium, Azospirillum and Azotobacter*), Phosphate solubilizing bio fertilizers (*Bacillus megathirium and Pseudomonas putida*), Phosphate mobilizing bio fertilizers (Mycorrhiza), Potash mobilizing bacteria (*Feratunia aruntia*), Zinc solubilizing bio fertilizer (*Bacillus, Pseudomonas and Thiobacillus*), Plant growth promoting biofertilizer (*Pseudomonas),* Sulphur solubilizer (*Thiobacillus thioxidans*), Silica solubilizer (*Pseudomonas fluorescens, Bacillus flexus).*

MATERIALS AND METHODS

Collection of sample

The soil samples were collected randomly from the rhizosphere region of mature coffee plants of different localities of Thandikudi, Dindugul District, Tamil Nadu, India. The samples were transferred aseptically to the lab through closed container.

Isolation of *Thiobacillus thioxidans* - Serial dilution technique

One gram of soil samples were taken and it was added to 10ml of sterilized distilled water to make serial dilution of 10-1 to 10-6.(Koch,1883) One millilitre of samples from 10-3 to 10-5 were spread on the Thiobacillus specific medium like Mineral salt agar, Bunt and Rovira agar or Thiobacillus agar medium. Finally the plates were incubated at 30˚C for 2 – 3 days. After incubation the colonies were isolated and it was transferred to Mineral salt agar slant and stored at 4˚C for further use (Premalatha et al., 2019).

Morphological Characterization of *Thiobacillus Thioxidans*

Morphological characterization was analyzed by plating methods using specific medium like Bunt and Rovira agar medium for *Thiobacillus* growth. After 48 hours, the grown cultures were subjected to morphological characterization.

Gram Staining

Gram stain is a differential stain. It needs primary stain and counter stain. Gram staining divided the bacteria in to two large groups such as Gram positive and Gram negative. The protocol was followed as per Colco, R, 2005.

Biochemical Characterization [Bergey's Manual of Determinative Bacteriology. 9th edition]: (Baltimore et al., 1984)

Catalase Test

This test is used to identify the organism which produces an enzyme catalase. The enzyme catalase breaks the hydrogen peroxide into water and oxygen gas. The production of air indicates the oxygen formation. It indicates the positive result of catalase.

$2H_2O_2 \rightarrow 2H_2O + O_2$

The isolates were grown in Bunt & Rovira broth and loopful of culture was transferred to the clean slide. One drop of hydrogen peroxide was added and results were observed.

Oxidase Test

This test is used to identify the microorganism which produces an enzyme Cytochrome oxidase. Cytochrome oxidase is important in the electron transport chain. The enzyme transfer electron from the electron transport

chain to oxygen and reduces it to water. During electron transport, the electron donor is oxidized by Cytochrome oxidase. The isolates were grown in Bunt & Rovira broth. A loopful of pure culture was taken and it was placed on the Oxidase disc.

Citrate Utilization Test
This test is used to differentiate the bacteria. The utilization of citrate by the test bacteria will produce alkaline by product. Simmon Citrate agar was prepared and poured it into the sterile test tubes. Isolates were inoculated and incubated to observe the colour changes.

Methyl Red Test
In this test the microorganism utilize carbohydrate from the MR broth and it will produce large quantities of acid. Methyl red is an indicator. The isolates were inoculated in MR-VP broth and incubated for 24hrs at 37°C for 24 hours. 5 drops of methyl red indicator was added to the culture and the results were observed.

Voges Prosker Test
This test used to differentiate an enteric organism. To the grown culture in MRVP broth, 0.5ml of alpha napthol and 0.2ml of KOH was added and the results were observed.

Indole Test
The test is used to detect the ability of micro organism to degrade the amino acid Tryptophan. Peptone water was prepared and the isolates were inoculated. After incubation at 37°C for 24 hours, 1 ml of Kovac's reagent was added.

Nitrate Reduction Test
The test is used to detect which microorganism involved in nitrate reduction. To the nitrate broth, the isolates were inoculated and incubated for 37°C at 24hours. After incubation, sulfonilic acid and alpha- naphthylamile was added to see the nitrate reduction.

Ammonia Test
In this test, nutrient broth was prepared and the isolated culture was inoculated and incubated at 37^0C for 3 to 5 days. After incubation Nesslew's reagent was added and the colour changes were observed.

Testing Of Biocontrol Ability - Antagonistic Test
Thiobacillus isolates was originally isolated from the soil of coffee plants grown in field soil and collected from different plant growing areas. They were cultured on zinc solubilizing agar at 28 °C for five days. After an incubation period, colonies were purified and determined to be *Thiobacillus* isolates (Rifai 1969). *Thiobacillus* isolates were grown in 50-ml ZSA broth (PDB,Difco), for 10 ± 2 days at room temperature. Colony was harvested by filtration through a piece of filter paper and washed with distilled water.

PLATE CONFIRMATION TEST
Plate confrontation assays of *Thiobacillus* isolated from coffee soil region were evaluated in vitro as antagonists against isolates of *R. solani* (pathogen). Dual cultures were carried out by using one-week-old cultures of *R. solani* and *Thiobacillus thioxidans* on Zinc Solubilising Agar. The agar medium was inoculated with a 5-mm-diameter disc of antagonist positioned diametrically opposite a 5-mm-diameter disc of the pathogen. The distance between discs was approximately 5 cm. The cultures were grown at 28 ± 3 °C, and measurements were taken after four days. In the control treatment, a sterile agar disc (0.5 mm diam) was placed in dish instead of *Thiobacillus* isolates. There were three replicates for each treatment. At the end of the incubation period, redial growth was measured. The efficiency of *Thiobacillus* isolate in suppressing radial growth was calculated as follows: **(C–T)/C × 100**

Where, C is radial growth measurement of the pathogen in the control and T is radial growth of the pathogen in the presence of *Thiobacillus* isolates.

Confirmation Test
The isolated colonies from Zinc solubilizing agar were further transferred into the specific media, Thiobacillus agar medium for the confirmation test. The inoculated plates were incubated at 25 - 30°C for 7 days.

Plant growth promoting activity
HCN Production
The HCN production method was detected by Bakker and Schippers [1987]. To the nutrient agar plates, the bacterial culture was streaked. Whatman No 1 filter paper was dipped with 0.5% of Picric acid and 2% of Sodium carbonate and then it was sealed with parafilm and incubated at 28°C for 4 days. The colour change of the filter paper was noted.

Siderophore Test
Bacterial Siderophore are produced by using inoculation of isolates with Succinate broth. The overnight culture was centrifuged at 1000rpm for 5minutes. Then 1ml of supernatant was added with 1ml of 2% $fecl_3$. Siderophore test indicates the gaining ability of irons from soil. If the isolated bacteria will produce siderophore (Uptake of Iron), the colour changes occurred in bacterial supernatant (Meyer and Abdullah, 1978).

Zinc Solubilization
An incubation study was conducted to evaluate the zinc solubilizing potential of isolated bacteria with ZnO. The solubilization potential of bacteria was assessed under *in vitro* conditions (Saravanan *et al.*, 2003). To check the Zn solubilization ability of selected strains, bacteria were subjected to grow on Bunt & Rovira media containing 0.1% insoluble zinc compound (ZnO) as described by (Bunt and Rovira, 1955). Pikovskaya media (Pikovskaya R.I, 1948) also used for zinc solubilization instead of Bunt and Rovira medium with the presence of

Bromophenol blue indicator. Bacterial cultures were spot inoculated on the media using a sterile loop. The Petri plates were incubated at 28±1°C for 5 days.

Formulation Of Solid Biofertilizer

The good quality of vermicompost and talcum were collected (50%Vermi+50%Talc+400ml of inoculum). Then it was converted into fine powder format. The carrier materials are sterilized to eliminate the contaminant. Then the isolated bacterial inoculum (10^{-9} CFU/ml) was added to the carrier material and shade dried for few hours. Temperature, pH and moisture of the carrier material for the survival of the thiobacillus isolates were maintained in every step. (Springer *et al*., 1994)

Formulation Of Liquid Biofertilizer

The strains used for liquid biofertilizer formulation was zinc solubilizing bacteria *(Thiobacillus).* It was cultivated in zinc solubilizing agar media or Bunt & Rovira agar media, and then it was incubated in rotary shaker at 30ºC for 24hours. After incubation, temperature and pH of the broth for the survival of the isolates were monitored. Then it was directly used as biofertilizer. The shelf-life of liquid biofertilizer was higher than others (up to 180 days) (Springer *et. al*., 1994).

Formulation Of Capsule Biofertilizer

The best quality of talcum was collected and mixed with bacterial culture and shade dried for few hours. After dried, it was packed in the Gelatin capsule with suitable physical parameters and the size of capsules such as 250mg, 350mg and 1000mg were used (Springer et al., 1994).

RESULT AND DISCUSSION

The primary purpose of writing this article is help to Coffee farmers worldwide in understanding the basic premise on which bacteria operate and the ways it means of carefully exploiting their potential to maintain a suitable ecological balance within the Coffee farm.

Figures 1: Isolates grown in Zinc solubilising agar medium.

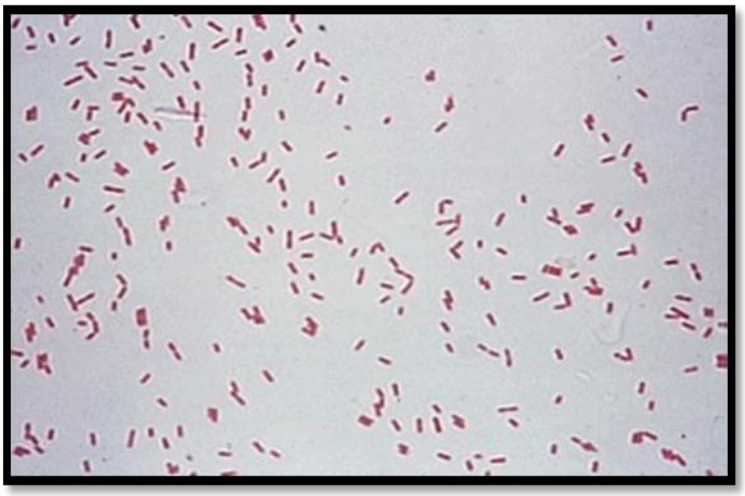

Figure 2: Microscopic view of *Thiobacillus thioxidans.*

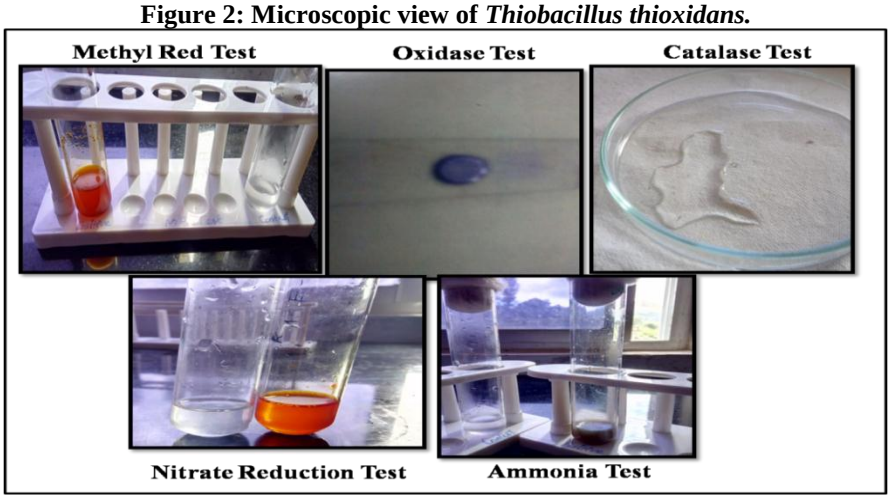

Figure 3: Biochemical characterizations.

Figure 4: Zinc solubilizing broth and plates.

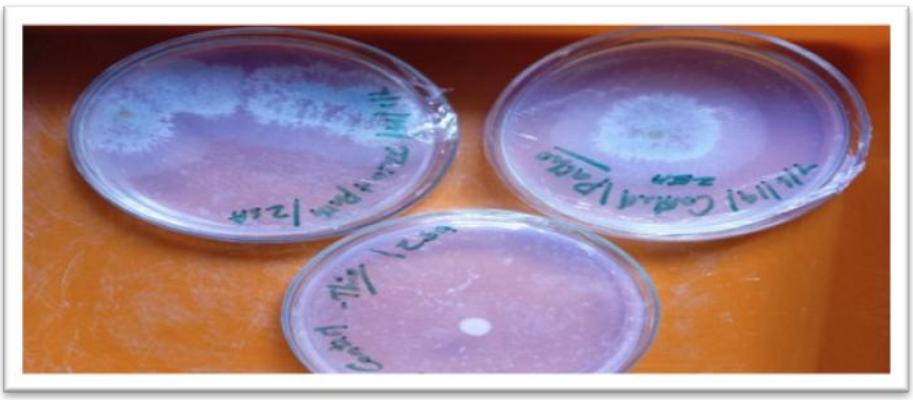

Figure 5: Antagonistic Activity.

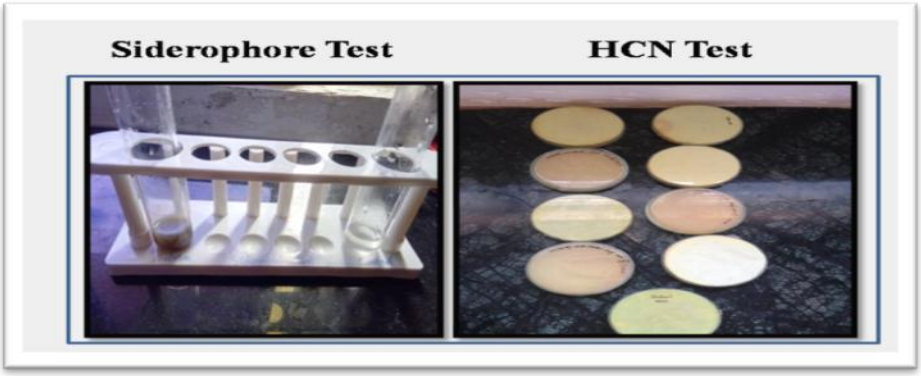

Figure 6: Plant growth promoting activities.

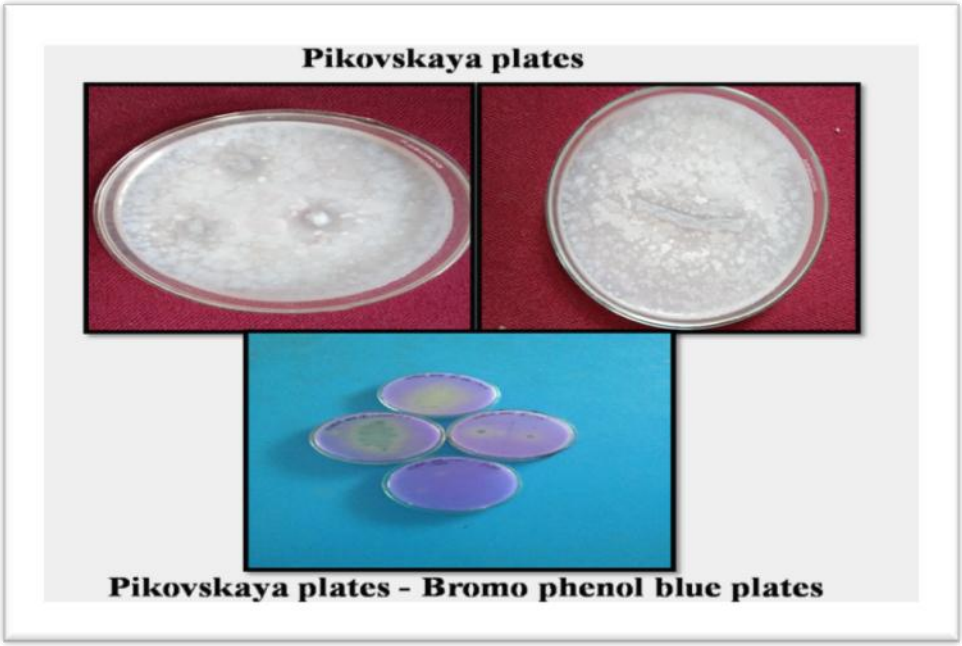

Figure 7: Zinc solubilization of *Thiobacillus* isolates

Figure 8*: Thiobacillus thioxidans* biofertilizer formulation

Table 1: Biochemical Test.

Sl. No.	Sample	Citrate test	Methyl red test	VP test	Oxidase test	Catalase test	Ammonia test	Indole test	Nitrate test	Motility test
1	TT1	+	+	_	_	+	+	_	+	+
2	TT2	+	+	_	_	+	+	_	+	+
3	TT3	_	+	_	+	_	+	_	+	_
4	TT4	+	+	_	+	_	+	_	+	+

TT – *Thiobacillus thioxidans*

Table 2: Plant Growth Promoting Activity.

SL. NO.	SAMPLE	RESULT
1.	HCN Production	+
2.	Siderophore test	+

Table 3: Zinc Solubilisation.

S. No.	Samples	Zone formation
1	TT1	1cm
2	TT2	0.8cm
3	TT3	0.7cm
4	TT4	1.5cm

Zinc solubilisation was increased in the field by spreading the *Thiobacillus* sp isolated from the rhizosphere soil region. In the present work, using Thiobacillus agar, the bacterial isolates were isolated from rhizosphere soil from different localities. White colour single or paired colonies were observed in Thiobacillus agar plates and slants (Saravanan *et al*., 2003). Results showed that more number of isolates were present in rhizosphere region compared to non-rhizospheric soil reported by Reyes *et al*., 2006 (Figure 1).

By using Gram's staining, the identification of morphology of the four isolates are observed. The isolated colonies from the Thiobacillus agar plates are Gram negative, rods (Colco, R, 2005) (Figure 2). Based on the Biochemical test, four colonies were identified as *Thiobacillus* sp. (positive result was obtained in Citrate utilization, Methyl red, Oxidase, Ammonia and Nitrate tests while negative result for VP and Catalase tests) and the confirmed isolates (TT1, TT2, TT3 & TT4) further used for this study (Baltimore *et al*., 1984) (Figure 3) (Table 1).

For the identification of growth of Zinc solubilizer, the isolated colonies were transferred to the Zinc solubilizing broth and Zinc solubilizing agar plates. White colour colonies colonies were observed and converted the broth to turbid and transparent. This shows that zinc present in the medium was solubilised by the isolates and so capable to grow in the specific agar plate and broth (Figure 4) (Saravanan *et al*., 2003).

Thiobacillus isolates were subjected to antagonistic characterization**.** A microbial biocontrol ability may express different mechanisms against plant pathogens during their antagonistic activity. It weakening or destroy the pathogen directly by the synthesis of antimicrobial compounds. In this study, the antagonistic effect was expressed by the *Thiobacillus* sp. in dual culture method. The agar medium was inoculated with a 5-mm-diameter disc of antagonist positioned diametrically opposite a 5-mm-diameter disc of the pathogen. At the end of the incubation period, radial growth was measured. The efficiency of *Thiobacillus* isolate in suppressing radial growth was calculated. Thus the results, Genus Thiobacillus act as a biocontrol agent to prevent some fungal disease in Coffee plants (Agrios, 2005) (Figure 5).

The plant growth promoting activities (PGPA) like HCN production and Siderophore test has been reported (Figure 6) (Table 2). HCN test cleared the production ability of Hydrogen, Carbon and Nitrogen by the isolated bacteria. Followed by the procedure of Bakker and Schippers [1987], the agar media poured Petri dish containing Whatman No.1 filter paper turns yellow to orange or brown colour due to the productions of HCN (Bakker and Schippers 1987). Siderophore test indicates the gaining ability of irons from soil. If the isolated bacteria will produce siderophore, the culture supernatant turns to deep orange colour with the presence of $Fecl_3$. Under iron limiting conditions, Thiobacillus produce siderophore for the purpose of iron uptake (Meyer and Abdullah, 1978).

Further confirmation of Zinc solubilisation by Thiobacillus was clearly reported by growing the isolates in Pikovskaya medium. The isolated colonies have an ability to solubilization of Zinc materials, it is clearly confirmed by formation of clear zone in the Pikovskaya agar plates and Pikovskaya – BPB (Bromo phenol blue) plates with the presence of ZnO. The zone measurements is tabulated in Table 3 (Saravanan et al., 2003) (Figure 7).

The isolates shelf – life ability was tested by three formulations such as solid, liquid and capsule bio fertilizers. In Solid or Carrier based biofertilizer (Vermicompost & Talcum powder) the inoculant containing effective isolates were used as biofertilizer preparation. The easy, long term storage and high effective of biofertilizer is enabled in carrier material due to incorporation of microbes. There are various types of biofertilizer present among them the bacterial inoculants is one of major group which includes rhizobia, nitrogen fixing *rhizobacteria*, plant growth promoting and phosphate solubilising, zinc solubilizing bacteria and so on. The bacteria which included in biofertilizer have close relationship with plant roots. "Seed inoculation'' is the most common way of inoculation in which inoculants (bacteria-carrier mixture) is mixed with water to make slurry-form and then mixed with seeds. (Springer et al., 1994).

After the proper preparation of fine powder with inoculants of each formulation, it was distributed in to the planting soil (Figure 8). The Liquid biofertilizer consists of direct inoculation of desired bacterial colonies without any carrier based. It has high shelf life than other fertilizers and easily portable from one place to another. Capsule formulation gives best result than other formulations. In Capsule biofertilizer formulation, the Gelatin capsule was used for preparation, due to its easy

handling, eco –friendly in nature, biodegradable, cheaper source and most importantly non-toxic to environment.

CONCLUSION

The present study reported the role of zinc solubilising biofertilizer. An overview of this study suggested three types of zinc solubilising biofertilizer formulations and shelf life periods. The genus level identification confirmed *Thiobacillus thioxidans* as the potential zinc solubilizer. Among the various forms of biofertilizer, capsule formulation is recommended to get better result than other formulations.

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