Discussion:

Plentiful isolates were isolated from tea rhizosphere of Barak Valley, Assam, India in four different seasons of the year 2009-2010. Soils from Ichabil, Bagbahar, Dwarbond and Silcoorie tea estates from clonal tea varieties were taken from a depth of 0-25 cms (centi meters). Before doing any isolation the soil analysis was carried out for pH, moisture content and N, P resolved. From the soil analysis test along with the availability of microbes present in the tea rhizosphere, it is found that P, N and C inputs of soil influence the microbial growth which is in agreement with the results mentioned by Singh et al. (1989). Also the seasonal changes, moisture content of soils and Carbon content of soil influences the microbial growth, that is in accordance with Ross (1987).

For listing of CFU counts individual mediums such as Nutrient agar for general bacteria (Anon, 1957), Martin agar (Martin, 1950) for fungi and Burkâ€™s medium (Subba Rao, 1977) for diazotrophs were used. Based on the CFU counts, F- Test was worked out, an ANOVA table was prepared and the level of significance was determined and found that results were significant. Other microbes isolated from the tea rhizosphere were tentatively identified as, the genera Bacillus and Pseudomonas as those microbes have been reported in rhizosphere of different plants earlier by Gupta et al. (2002); Chakraborty et al.(2005); Mishra et al., (2005). Finally, all isolates were identified by following Bergeyâ€™s Manual of Determinative Bacteriology (Holt et al., 1994). On the basis of CFU counts of microbes (diazotrophs, bacteria and fungi), maximum growth of micro flora around tea rhizosphere was seen in the month of July 2010, followed by August 2009 i.e. in monsoon and pre monsoon seasons in Assam, this may be due to high moisture content and nitrogen content available in the soil. Lesser microbial growth was observed during the winter seasons September 2010 and January 2010, i.e in post monsoon seasons, which was in accordance to the results carried out by Shilpkar et al. (2009).
For Okon isolates:

The isolates obtained on semi solid nitrogen free medium were looked curved rods, with possessing motility the results were in accordance with Kreig (1976). The isolates grew best at temperature around 20 and 30°C. Except the isolate Oko2, rest all the isolates utilized 1% NaCl, the isolates obtained had a wide range of size between 1.1-1.7μm, this was in accordance with the similar result mentioned for the isolates in Bergey’s Manual 9th edition (Holt et al., 1994) for different strains of Azospirillium, this has been also mentioned by Usha et al. (2011) for similar isolates of saline tolerant Azospirillium strains from paddy field of Thanjavur district. The isolates showed variable carbon utilization; this was agreement to Usha et al. (2011). Also showed positive response towards antibiotics tetracycline and chloromphenical. Regarding their response to pH; some of the isolates grew at pH8, some at pH4 and all isolates grew at pH6. As all the isolates grew at pH6 this result was in accordance with the similar type of results for similar type of isolates obtained by Eckert et al. (2001). Colour of Oko isolates obtained is as per the similar isolates obtained by Tennakoon (2007). Regarding biochemical traits, the isolates were seen to be Gram negative rod shaped bacteria that result got matched for the similar isolates obtained by Usha et al. (2011). The isolates showed negative gelatin hydrolysis, negative MR-VP reaction, the results were in accordance to the results obtained by Jakhar et al. (2011) for the isolates of genus Azospirillium obtained by them. All Oko isolates gave positive urease test. Variable starch hydrolysis were shown by the Okon isolates as isolate numbers Oko1, Oko2 and Oko4 did not hydrolyzed starch, the fact was compared with the results obtained by Usha et al. (2011). As after comparison it was found that out of the ten Azospirillium isolates obtained by Usha et al. (2011), two isolates could not utilize starch, rest undergone starch hydrolysis. Regarding the catalase test, oxidase test and nitrate reduction, all the seven Okon isolates gave positive result for the catalase, oxidase and nitrate reduction. Based on the above mentioned features the isolates were identified to its genus level. The ARA (Acetylene Reduction Assay) for an isolate was done, that may be the amount is low but still it may be due to enzymatic activity, as all reactions are enzyme controlled. So the test for ARA
revealed its nitrogen fixing capability; this was in accordance with the results obtained for similar isolates obtained by Veena (1999) and Naiker (2003).

Isolates obtained on YEMA medium:

Two isolates were chosen from seven isolates that has been obtained from YEMA (Yeast Extract Mannitol Agar) medium and they were tentatively identified up to their genus level initially. And later by 16S rDNA sequencing for an isolate having the better potential of nitrogen fixation was selected and identified as Rhizobium sp. Based on nucleotide homology and phylogenetic analysis it was found that the species is of the genus Rhizobium sp. CL4.3 strain. Finally the accession number for that bacterium was attained as KC247672.

It was observed that, the isolates appeared as white in colour in the medium; they were Gram negative in nature and rod shape in appearance under microscopic. The strains nicely utilized 1% NaCl, they grew best at the temperature of 30°C, also they grew best at pH 6 to 8, no growth was observed at pH 4 and 9, also above 30°C no growth was seen, this was similar to the result obtained for the Rhizobium isolates obtained by Singh et al. (2008). Similar result have been obtained for the strains that grew at around 30°C, being said by Kucuk et al. (2006) that too was in accordance with the results obtained for the isolates of Rhizobium obtained by Singh et al. (2008). The two Rhizobium strains easily utilized the different mentioned carbon sources that were in accordance to the similar result for the similar isolates obtained by Singh et al. (2008). The only exception was that, the strains could not utilize citrate as the carbon source that result was similar to the result obtained by Joseph et al. (2007) for his strains that belong to the genus Rhizobium. For the biochemical characters; the isolates showed positive MR-VP reactions, positive starch hydrolysis, catalase positive, oxidase positive, they showed nitrate reduction, but did not undergo gelatin liquefaction and were found to be of motile nature ,the similar facts have been mentioned for the Rhizobium isolates by Joseph et al.(2007). The isolates grew best at pH6 also they grew at pH8. And the obtained strains of Rhizobium showed susceptible towards tetracycline and chloromphenical. Finally after doing the 16SrDNA sequencing the species level identification was achieved for a strain. The strain was identified as Rhizobium sp. CL4.3 that was achieved by finding the close homology of the bacterial sequence after carrying out BLAST.
The two isolates were found to possess a size of 2.2 and 2.4 μm. The nitrogen fixing ability of an isolate was depicted by ARA (Acetylene Reduction Assay) that signifies that it is nitrogen fixer. The nitrogen fixing ability of the isolate is in agreement with the similar *Rhizobium* isolate obtained from different chick pea growing regions of peninsular India by Rai *et al.* (2012).

**For Luria Isolates:**

The isolates on LA (Luria Agar) medium was isolated from tea growing regions, out of several isolates, five best isolates were picked and carried out for the different analysis. From the external appearance it appeared that the LA strains were of white colour, the colonies were round and of Gram positive nature, tentatively on the basis of that they were identified as *Bacillus* spp. as per *Bergey’s Manual of Determinative Bacteriology* (Holt *et al*., 1994). The isolates obtained on LA (Luria agar medium) showed the following biochemical features as were found to showed nitrate positive reduction test, catalase positive, oxidase negative, all gave positive test for starch hydrolysis, strain number LA1, 2 and 4 showed gelatin liquefaction, isolate LA1, LA2, LA3 and LA5 showed positive urease activity, all isolates showed positive response towards MR (Methyl Red Test) and none with VP activity. Physiologically, the isolates grew ideally at 30°C and at pH 6; also they showed the capacity of 1% NaCl tolerance power. That was Similar to the results for the similar isolates of *Bacillus* obtained by Karimi *et al.* (2012) were also Gram positive, NaCl tolerance up to 7%, by his isolates of *Bacillus*. Regarding the carbon utilization the isolates used all carbon sources; dextrose, sucrose, citrate and mannitol same as the similar *Bacillus* isolates obtained by Joseph *et al.* (2007). All LA isolates were of non motile nature, they were sensitive towards antibiotics like chloromphenical, Gentamycin and Tetracycline.

**Isolates obtained on the King’s B Agar Medium:**

The isolates obtained on the King’s B agar medium (King *et al.*, 1954) were recognized to approximation to genus level based on their appearance and other traits. The isolates that were obtained on the King’s B agar medium were Gram negative rod shaped bacteria when seen under microscope of size ranging from 1.7 to 2.4 μm, they were observed to be of motile nature and white in colour. All the isolates utilized 1% NaCl, growth maximum was observed at 20°C. Except for a few isolates that grew at a pH 4, rest of the isolates the maximum growth was seen
at pH 6 and pH 8. Regarding the utilization of carbon sources by the isolates, it was seen that the isolates used all the three carbon sources except mannitol.

The carbon utilization patterns were found to be exactly similar for the results obtained by Joseph et al. (2007) for the similar isolates obtained by them. Likewise, the biochemical tests of the isolates obtained on the King’s B agar medium showed the following results: they were observed as Gram negative bacteria, showed positive catalase test, oxidase positive test, nitrate reduction positive by all the isolates, some of the isolates showed starch hydrolysis whereas some did not respond towards starch hydrolysis, the results were found to be similar that for the similar isolates obtained by Karimi et al. (2012). The isolates obtained so were found to give negative response towards gelatin liquefaction and did not showed any reaction towards MR VP test, this was in agreement with the results obtained by Joseph et al. (2007) for similar isolates obtained by them. None of the isolates showed urease activity. The isolates were tested for antibiotic sensitivities and showed nice results. From the morphological appearance, physiological studies at different temperature conditions, pH, and salt conditions and also on the basis of the biochemical tests the isolates obtained on the above said medium were recognized as of the genus *Pseudomonas*. This was done by following the Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994), although the 16S rDNA analysis for the isolates was not been carried out.

**Isolates obtained on Ashby sucrose medium:**

For the isolates obtained on Ashby sucrose medium were of rod shape, motile nature, transparent dew drop like colonies with a size ranging between 1.2-2.1 μm. As the colonies were seen as smooth, opaque, motile and Gram negative rods, that were found close approximation with the genus *Azotobacter* on comparison with the Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994). The isolates grow best at 30°C; except for the isolate number 6 that did not grow at 30°C temperature. Some of the isolates like Azo1, Azo3, Azo5 and Azo6 grew at 20°C; all the isolates gave tolerate for 1% NaCl, except for the isolates Azo1 and Azo8 which did not show tolerance for 1% NaCl. It was observed that their best growth occurred at pH 6, isolate number 8 could not grow at pH 8 rest all grew at that pH. Also some of them could grow at a
Regarding the response of the isolates towards the carbon sources all the isolates utilized carbon sources: glucose, sucrose and mannitol but only two isolate showed positive response towards citrate, they were isolate number 6 and 7. All the isolates showed susceptibility towards antibiotic chloromphenical and mixed response towards tetracycline.

Response of isolates towards biochemical reactions were the following: all the isolates were Gram negative, catalase positive, oxidase positive and nitrate positive, negative response towards urease and gelatin liquefaction tests. The biochemical traits were in accordance with Naz et al. (2012) as their isolates of *Azotobacter* spp. showed catalase positive result, oxidase positive result, and negative result for VP test, also utilized carbon sources glucose, sucrose and the result were similar to the results for the similar isolates obtained here in this work. The results here showed that none of the isolates showed any response towards urease activity as well as tests for VP which was in agreement to the results of similar isolates of *Azotobacter* by Chavada Nikul et al. (2010). Also as here the isolates utilized mannitol as carbon source and a few used citrates as carbon source, two isolates Azo12 and Azo13 showed positive Methyl Red test (MR), this is in agreement with the results obtained by Nagananda et al. (2010) for their similar type of isolates of *Azotobacter*. The biochemical test done for the isolates obtained on Ashby’s medium was compared with the result obtained by Joseph et al. (2007) for similar isolates of *Azotobacter*. The *Azotobacter* isolates obtained by Joseph et al. (2007) gave positive starch hydrolysis, positive nitrate reduction, positive oxidase and catalase action, also were Gram negative nature and rod shape in appearance when seen under a microscope. They found that their isolates of *Azotobacter* have used glucose, sucrose and mannitol as carbon sources which again were in accordance with the results obtained for thirteen isolates obtained here in this study on diazotrophs from tea rhizosphere of South Assam, India.

As 16s rDNA sequencing method has become an indispensable tool for species level recognition of the isolates, so on the basis of 16s rDNA sequences a total of three Azo isolates on the basis of their nitrogen fixing ability were chosen.
The culture marked as AZO-2 was found as *Azotobacter armeniacus strain DSM 2284* (GenBank Accession Number: KC137338) on basis of nucleotide homology and phylogenetic study. The culture labeled as AZO-3 was found to be *Azotobacter salinestris strain ATCC 49674* (GenBank Accession Number: JX680337).

The culture, that was marked as AZO-4 was found to as *Azotobacter beijerinckia strain ICMP 8673* (GenBank Accession Number KC172855 on basis of nucleotide homology and phylogenetic analysis.

On the basis the 16s rDNA sequences of the isolates , the species level identification was confirmed , the isolate marked as AZO-2 was *Azotobacter armeniacus*, similarly AZO-3 was found to be as *Azotobacter salinestris*, AZO-4 as *Azotobacter beijerinckia* and a strain marked as Rhiz-live5 was of *Rhizobium* spp. And finally Phylogenetic trees were constructed by taking each above mentioned isolates obtained from the tea rhizosphere of south Assam, India along with ten reference isolates against each above mentioned isolate, that revealed the species level identification of the isolates. A total of four Phylogenetic trees were prepared with each strain along with ten reference strains taken from Gen Bank of NCBI. The Nitrogen fixing ability of some of the isolates have been portrayed by ARA, which proved their ability for nitrogen fixation.

Molecular tools in amalgamated with microbial methods will lend a hand to us for exploring their genetic multiplicity. Among the different isolates obtained from the tea rhizosphere of different tea growing gardens of South Assam, the diazotrophs of genus *Azotobacter* and fungus of the genus *Aspergillus* were found to be predominant in all conditions.

The soil parameters along with physiological, morphological, biochemical and molecular aspects of the isolates obtained from tea rhizosphere were taken into account in this investigation. After collecting soil samples from different tea gardens (Ichabil,Dwarbond, Bagbahar and Silcoorie) of Southern Assam, India in different seasons the soil parameters pH,moisture content N,C,K was calculated, and it was found that the soil in this region is of acidic nature and soil is sand loamy to clayey.
A total of 39 bacterial isolates were collected from the rhizosphere of tea plants of Southern Assam, India in four different seasons from the clones of varieties (Toklai Variety) TV17, TV20, and TV23 in the year 2009-2010.

As 16S rDNA sequences are highly conserved among the different species of same genera and not prone to mutation, so it is used as an integral method for species level identification. A total of four phylogenetic trees were constructed and phylogenetic positions were located. The amounts of nitrogen fixed by the *Azotobacter* isolates were in accordance with the results of Ashraf *et al.* (2011) obtained for similar isolates.

**The Biocontrol Potential:**

The biocontrol actions of some of the best isolates were chosen from a few Luria isolates (*Bacillus* spp.) and some of the isolates of genus *Pseudomonas* spp. were chosen on the basis of effectiveness of their action for suppression of *Fusarium* growth. The mechanisms by which they inhibit the fungal growth were displayed, such as production of HCN (Hydrogen Cyanide) by the isolates or volatile metabolites were identified. From this work, three *Bacillus* isolates were selected and seven *Pseudomonas* isolates were chosen and their mechanistic roles were displayed. From that it has been found that three *Bacillus* had suppressed the mycelium growth of *Fusarium* and that was due to the production of volatile metabolites, similarly the seven *Pseudomonas* isolates were displayed for their antagonisms against *Fusarium* and seen that none displayed HCN production and it was due to volatile metabolite production but from that also two isolates did not show the volatile metabolite productivity, so it may be due some other unknown mechanisms. Regarding the biocontrol role played by *Bacillus* spp. against *Fusarium* sp. was compared and found that some *Bacillus* spp. isolates inhibited the mycelium growth of *Fusarium oxysporum* which is in accordance to the results of Karimi *et al.* (2012). The biocontrol activity by *Pseudomonas* spp. against *Fusarium* sp. was in agreement to their results obtained by Velusamy *et al.* (2011) . A minor statistical analysis was carried out on the biocontrol activity tests of the ten bacterial isolates against fungi, from that the zone of inhibition was calculated.

The role of biocontrol potential action of *Bacillus* spp. was determined and elucidated against *Fusarium oxysporum* and the results were found similar as been mentioned by different
The biocontrol role played by \textit{Pseudomonas} against \textit{Fusarium oxysporum} was satisfactory and was similar in results obtained by for similar non fluorescent pseudomonas sp. against \textit{Fusarium oxysporum}.

As we know that tea is the most extensively consumed beverage by the people across the globe and from the ancient times, it had been regarded as the supreme beverages among other beverages, mainly in India. It is a health conducing drink, conspicuous for promoting health to humans. In Barak valley after rice, tea is next grown in this region and considered as the plant of most economic importance. As the population of the world is increasing at an alarming rate and in order to give food to the constantly increasing population, the farmers are using chemical fertilizers, insecticides and pesticides to control the plants as well as crops of being getting damaged by the pests and insects and to have more productivity. But the over use of pesticides ,chemical fertilizers and manures for increasing the crop yield are directly or indirectly affecting the environment as well as human beings and other organisms. As those toxic pesticides and fertilizers once being applied to the plants are consumed by humans as a result that starts accumulating in the body, which have obnoxious affects, also they find their ways to water bodies like river, ponds, lakes and other water bodies, once being carried away by rain. That has resulted in eutrophication problem that has lead to the BOD (Biological Oxygen Demand), as more the BOD the more will be the level of pollution in water bodies. Similarly, the accumulation of toxic particles have led to biomagnifications which is nothing but the accumulation of toxic materials in the food chain and by that phenomenon the toxic materials starts accumulating from one trophic level to the next trophic level and finds their way to human bodies. As a result the incidence of life threatening diseases like cancer have increased over the last decade or so on. So, researchers and scientists across the globe has come up with an idea for a natural way for production of crops which is known as organic farming, that includes biological nitrogen fixation, biofertilizers, biocontrol potentials etc. with the help of microorganisms that are found in the rhizosphere of plants and trees that provide essential nutrients to the plants ,also some of them offer protection to plants and leaves by providing in antagonism against other pests and pathogens ,more prominently the above mentioned methods are safe methods of food production and also a clean and harmless approach which doesn't harm
human beings, animals and plants. It also lends a hand for the plants to fix nitrogen in a normal way. The connotations of the organisms found in the rhizosphere have been reported by numerous workers from time to time. The implication of those microorganisms present in the rhizosphere have been utilized for the advancement in the maturity of plants and for controlling the diseases in plants, have been emphasized by workers like Barka et al. (2000) and Chakraborty et al. (2005). In this study, an inquiry was carried out in four different tea cultivated regions of South Assam (Barak Valley): Bagbahar, Silcoorie, Ichabil and Dwarbond tea gardens of Southern Assam, India in four different seasons from the year 2009 August to 2010 September for segregation of some fresh strains of bacteria encompassing the capability for fixing atmospheric nitrogen and some having the biocontrol potentials. Thirty nine best isolates were screened out from a number of isolates from tea rhizosphere and categorized into their genera on the foundation of physiological, morphological and biochemical attributes. And lastly 16S rDNA sequencing was carried out for a few isolates and based on that phylogenetic trees were made. The entire study was based on the exploration that was made in 2009-2010.

Out of 39 strains, bacteria of the following genera: \( \text{Azotobacter} \) spp., \( \text{Azospirillum} \) spp., \( \text{Rhizobium} \) spp. \( \text{Bacillus} \) spp. and \( \text{Pseudomonas} \) spp.) were identified on the basis of their culture characteristics, morphology and biochemical characteristics to their genus level. Fungi of the genus \( \text{Penicillium} \) spp. and \( \text{Aspergillus} \) spp. in the tea rhizosphere were identified; this is achieved by following the identification protocols described by Gilman (1957). Mostly \( \text{Azotobacter} \) spp. are predominant in all the conditions in tea rhizosphere of Barak Valley, Assam, India. The above said research is really an interesting step in this region.