Chapter 1

Introduction

Soil microbial diversity is an unseen international resource that deserves greater attention. Despite of their abundance, the impact of soil microbes on ecosystem processes is still poorly understood. One gram of soil may harbour up to 10 billion microorganisms with thousands of different species (Roselló-Mora and Amann, 2001). Due to the complex community assembly at different levels of biological organization, less than 1% of the soil microorganisms observed under the microscope is cultivated and characterized; the rest are still remain uncharted. The study of genetic inconsistency within the microbial community is still an important event to encompass. High genetic variability within the taxons (species), species richness/evenness and wide range of functional groups (guilds) always draws a greater attention from the researchers all over the world. The diversity within the ecosystem level may be due to interactions between different ecological condition processes as well as a number of trophic levels. Therefore, analysis of microbial diversity from complex soil ecology should include multiple methods to measure their total community level and partial approaches targeting structural or functional subsets (Johnsen et al., 2001; Kozdrój and van Elsas, 2001).

Total genetic variation within a complex community is directly related to the amount of the distribution of information among their associates. The community
DNA assessment is now proved to be an important tool for investigation of genetic complexity within the microbial communities. The molecular techniques for community assessment are purely based on PCR amplification. Diversity of amplified sequences is simply resolved by differential electrophoretic migration on agarose or polyacrylamide gels, which depend on their size (ARDRA, t-RFLP, RISA, RAPD) or sequence (DGGE, TGGE). Complex banding patterns were generated, representing the genetic structure of the bacterial community. The profile data can then be analyzed in terms of similarities and relationships through dendrogram construction. Besides the fingerprinting techniques, sequencing of different housekeeping genes (16S rDNA, atpD, dnaK, gap, glnA, gltA, gyrB, pnp, recA, rpoB and thrC etc.) is very sophisticated approach to investigate the bacterial community composition within soil micro flora, due to their consideration as the evolution chronometer. New sequencing technologies such as pyrosequencing (so called 454 technologies) have been successfully used as rapid and efficient tools to enable in-depth analysis of bacterial composition and diversity of consortia of environmental microbes (Carriço et al., 2013).

Bacteria are by far the most diverse group of organisms exhibiting high levels of variability of genome content and structure, metabolic properties, cellular structure as well as lifestyles even within the species (Ochman et al., 2000). They can be found free-living or attached to the surface of soil particles in bulk soil; but a large number of soil bacteria are also found to be interacting with the roots of plants, in what is termed the rhizosphere. The plant rhizosphere province has been recognized as ‘hot spot’ for microbial colonization and activity due to persistent release of organic compounds from the actively growing root surfaces (Bowen and
Rovira, 1991; Chaparro et al., 2013). Among the rhizosphere bacterial community, fluorescent pseudomonads frequently dominate the soil rhizosphere, executing crucial role in nutrient cycling, soil fertility and plant growth promotion activity (Bergsma-Vlami et al., 2005; Hayat et al., 2010; Kumar et al., 2012).

Fluorescent pseudomonads are a very diverse group of rhizobacteria with 128 different species composition (Peix et al., 2009). The genetic diversity found within them gives rise to a wide range of phenotypes. Evidences depict the story of their wide range of diversity in genome architecture (chromosomes and accessory genetic elements) (Jablonka et al., 1998; Kirschner and Gerhart, 1998; Gogarten et al., 2002). Comprehensive DNA fingerprinting data analysis of fluorescent pseudomonads revealed a high level of polymorphism among different strains within a single species (Rainey et al., 1994; Ginard et al., 1997). Schmidt et al. (1996) reported genome size polymorphism from 5.2 to 7.1 Mbp among different strains of Pseudomonas aeruginosa. Ecological as well as genetic factors may imply a synergistic effect on genetic diversity within the ubiquitous bacterial group. (Spiers et al., 2000). The genetic composition of fluorescent pseudomonads is considerably different among the rhizospheres of different plant species cultivated in the same soil (Lemanceau et al., 1995; Bergsma-Vlami et al., 2005). Since they have promising plant growth promoting activity (Saikia et al., 2003; Aliye et al., 2008; Hayat et al., 2010; Kumar et al., 2012; Drogue et al., 2013) an exploration of their genotype diversity and metabolic versatility it is a very imperative obsession to be accomplished.

The functional diversity of fluorescent pseudomonads in terms of their diverse physiology was first confined by Stanier et al. (1966). They reported the diverse
group as a potential degrader of a wide range of substrates including aromatic compounds, halogenated derivatives and recalcitrant organic residues. Utilization of more than 100 different compounds as sources of carbon and energy clearly proves their diverse physiology (Madigan et al., 1997). Because of their catabolic versatility, metabolic products of fluorescent pseudomonads have been successfully used in different industrial field and found to be a good enhancer of industrial economic growth (Lynd et al., 2008). Besides the wide spectrum of industrial applications, their potential use in green agriculture has drawn the attention of research workers all over the world. Plant rhizosphere soil encompasses both beneficial and deleterious indigenous microbial populations; influences the plant growth and development, managing soil and plant health, and affecting the crop productivity in general. The plant beneficial microorganisms that colonize the root surface and the closely adhering soil interface were termed plant-growth promoting rhizobacteria (PGPR) (Kloepper and Schloth, 1978). Among the aboriginal rhizobacterial habitants, fluorescent pseudomonads are often considered as dominant rhizobacterial population and established their position as a potent biofertilizing and biocontrol agent (Haas and Défago, 2005; Ayyadurai et al., 2007; Weller, 2007; Salman et al., 2010; Kumar et al., 2012; Drogue et al., 2013). Biocontrol activity of fluorescent pseudomonads against the plant pathogens is often related to the production of a broad spectrum of extracellular lytic enzymes, siderophores, diverse antibiotics, hydrogen cyanide (HCN), or by activation of plant defense-responses (Saikia et al., 2003; Jamali et al., 2009; Zamioudis et al., 2013). Again, the efficiency for the production of different antibiotics such as phenazine-1-carboxylic acid (PCA) (Mavrodi et al., 1998; Wang et al., 2011; Du et al., 2013),
pyocyanin (Watson et al., 1986; Chai et al., 2013), 2-acetamidophenol (Slininger et al., 2000), pyrrolnitrin (Arima et al., 1964; Park et al., 2011), pyoluteorin (Howell and Stipanovic 1980, de Werra et al., 2011), phenazine-1-carboxamide (Chin-A-Woeng et al. 1998; Mavrodi et al., 2001a), 2,4-diacytethylphloroglucinol (Frapolli et al., 2012; Maketon et al., 2012, Asadhia et al., 2013), viscosinamide (Nielsen et al., 2000) and tensin (Nielsen et al., 2000) in different species of fluorescent pseudomonads has concavely upgraded their position as important biocontrol agents. Genes encoding the production of these antibiotics in fluorescent pseudomonads have been already identified, cloned and characterized (Mavrodi et al. 1998; Bangera and Thomashow, 1999; Hammer et al., 1999; Thompson et al., 1999; Mavrodi et al., 2001). Further, direct promotion of plant growth entails either production of the phytohormones such as auxins, cytokinins, gibberellins, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase or improvement of nutrient availability through a symbiotic N$_2$ fixation and phosphate solubilisation (Gyaneshwar et al., 2002; Glick et al., 2007; Selvakumar et al., 2013). Besides the application as PGPR and biofertilizers, their role in plant growth promotion under abiotic stressed condition has been exclusively studied. Fluorescent pseudomonads are reported to improve plant performance under stress environments and, consequently, enhance yield both directly and indirectly (Dimkpa et al., 2009). The ACC deaminase activity of fluorescent pseudomonads can induce stress resistance in plants lowering the stress-induced ethylene production by the course of degradation of ethylene precursor ACC (Glick et al., 2005).

The genetic diversity and functional characterization of this large group in rhizosphere soils of plants viz., rice (Naik et al., 2008), cotton (Hu et al., 2005),
banana (Ayyadurai et al., 2007), wheat (Thomashow et al., 1990; Yadav et al., 2013), canola (Patten et al., 2002), ground nut (Asadhia et al., 2013), sugarcane (Santhaguru and Saravanan, 2012; Nakade, 2013) have been already reported in different parts of the world. In North East India very few works have been reported on this aspect (Thakuria et al., 2004; Saikia et al., 2011; Tanti et al. 2010; Sarma et al., 2012). Therefore, exploration of genetic variability and functional characterization of this group from the Indo-Burma Mega hot spot region (Myers et al., 2000) through molecular approach assumes to be of greater importance.

Plants always responds to a wide array of abiotic and biotic factor in the environment such as - heavy metal action, wounding, water stress (draught or flooding stress), salinity, changes in temperature/light and pathogen/pest attack. Among the abiotic stress environments, water stress is seems to be one of the important factors for the reduction in crop productivity globally. Declining fresh water resource is one of the central challenges for improving food security across the world. Continuous negative impulses of climate change incorporate fur long competition for global water resources that increases vulnerability to food insecurity, particularly in Africa and Asia. More than 2 billion people, does not have access to clean water or sanitation (World Bank Statistics). With the current water management practices, by 2050 the global agricultural sector will need to double the amount of water used to feed the world, as water supply becomes scarce in some areas and more subject to extreme variations (Ziska, 2011).

The abiotic stress leads to a morphological, physiological, biochemical as well as molecular changes (Mayak et al., 2004). Water stress, salinity, extreme temperature and oxidative stress induce cellular damage to the crop by disruption of
homeostasis, ion destabilization in the cells and denaturation of functional and structural proteins (Saravanakumar et al., 2007). Such environmental stresses often activate certain cell signaling pathways and cellular responses i.e., production of stress proteins, upregulation of antioxidants and accumulation of compatible solutes (Mayak et al., 2004; Glick et al., 2007; Bajguz et al., 2009). Considerable progress has been made in understanding the molecular, physiological and morphological mechanisms of bacterial mediated tolerance to abiotic stresses (Van Loon et al., 1998). The control mechanism for abiotic stress resistance is purely based on activation as well as regulation of a definite set of stress related genes and helps the plant to escape from the stressed environment. Therefore, the induction to express proteins that can improve the physiology of suffering plant through beneficial microbes will be better for the plant as well as the environment (Saravanakumar et al., 2011).

The introduction of many stress-inducible genes via gene transfer resulted in improved plant stress tolerance (Zhang et al., 2004; Umezawa et al., 2006). Recently, a number of stress-inducible genes have been identified using microarray analysis in various plant species, including Arabidopsis and rice (Shinozaki and Yamaguchi-Shinozaki, 2007; Zeller et al., 2009; Pandey and Kim, 2012). Thus, analyzing the functions of these genes is critical to further our understanding of the molecular mechanisms governing plant stress response and tolerance, ultimately leading to enhancement of stress tolerance in crops through genetic manipulation. However, the transgenic approach for alleviation of abiotic stress condition is seemed to be quite cost effective. Again, the transgenic approach for crop improvement is still a matter for debate. Hence, application of an alternative eco-
friendly approach seemed to be acquiring a greater attention. Consecutive research on plant-microbe interaction, established the fact that, some of the bacterial strain having the property of mitigating biotic stress has also shown to protect plants against abiotic stresses. Thus for environmentally sustainable agricultural systems, bacterial inoculates providing cross protection against both biotic and abiotic stress would be highly preferable. Some workers had already reported the capability of fluorescent pseudomonads withstanding the abiotic stress suffering by different plants (Saravanakumar et al., 2007; Ali et al., 2009; Bano et al., 2009; Sandhya et al., 2009). A number of works have been reported on inducing draught tolerance capability in plants using Pseudomonas (Wright et al., 2003; Sandhya et al., 2009; Saravanakumar et al., 2011). However, so far very little has been done to explore the vast microbial genetic resources from north eastern part of India for useful agricultural applications. The acidic soils of the region may sustain a rich microbial repository that can be beneficial for plant health promotion. Therefore, our work was further extended to screen for potential bacterial strain(s) associated with mung bean, an important pulse crop of the region, for their potential effects on the alleviation of drought stress.

The economy of India mainly depends on agriculture. Indian agriculture is mainly depending upon two monsoon rains - South West monsoon and North East monsoon. With the 'Tropical Monsoon Rainforest Climate', Assam is a temperate region and experiences heavy rainfall and humidity, which causes flooding in Assam. Every year large areas of Assam come under the grip of floods, which causes extensive damage to crops. Similarly, in winter season the Ravi crops
exhibits the extreme water stress condition consequently crop productions are reduced significantly (Sharma, 2002).

Among the angiospermic world, Leguminosae or Fabaceae is the third most populous family after Asteraceae and Orchidaceae with 670 to 750 genera and 18,000 to 19,000 species. After cereal crops, legumes are the most important food crops based on total harvested area and production. India is the world’s largest producer of pulses, followed by Canada, Brazil, China, US, Mexico, France and Russia. In India, pulses incorporate the chief source of dietary proteins. Among pulse crops, the green gram (also known as mung bean) \([Vigna radiata \,(L) \,R. \,Wilczek]\) is an important crop in India. It is believed that green gram is a native of India and Central Asia and grown in these regions since prehistoric times. Besides an important source of human food and animal feed, green gram also plays an important role in sustaining soil fertility by improving its physical properties and fixing atmospheric nitrogen.

Unlike other crops, pulse crops are very sensitive to biotic as well as abiotic stresses. Besides other biotic as well as abiotic constraints, both high as well as a low rainfall affect the pulse crop adversely, which may have the adverse effect on the farmer’s hope of getting remunerative prices, even though consumer prices are high. Drought stress was recorded as the major limiting factor for pulse production in the Asia-Africa-Oceania regions (Johansen et al., 1992), North America (Slinkard et al., 1992) as well as in the Europe (Monti et al., 1992). In India, green gram was grown over an area of 9668 hectares with a production of 853kg per hectare in 2010-2011 (Agricultural Statistics Division, Directorate of Economics &
Statistics Department of Agriculture & Cooperation, Govt of India). In Assam, the production of green gram is about 459 kg per hectare in 2005-2006 (Ministry of Agriculture, Govt. of India). Jorhat district is one of the important producers of green gram with a cultivation area of 21.89 hectares with production of 600 kg per hectare during 2007-2008; however all the green gram cultivation areas were declared as rainfed (ACPDJ, 2012) that about 87% area under pulses is rainfed and consequently pulses face severe moisture stress with low productivity. Total area under the cultivation of pulse crops in Assam (as percentage of total cropped area within the state) was assorted marginally from 3.4% (1960 - 61) to 3.8% (1980-81) and then again reversed to 2.8% in 2005-2006 (Department of Agriculture, Govt. of Assam). In case of pulses taken together, the yield rate per hectare for Assam gradually decreased from 589 kg in 2000-2001 to 520 kg in 2005-2006 due to water stress condition (Status Report, Department of Agriculture, Govt. of Assam), whereas the same rate in India gradually increased from 574 kg to 616 kg. One of the main causes for this low production of pulse crop is due to water stress experienced by the crop every year (Sharma, 2002). The water stress experienced by the crop can be minimized through application of plant growth promoting bacterial communities (Saravanakumar et al., 2011). Thus, the identification of beneficial rhizobacteria along with biochemical and molecular mechanisms of interaction for water stress resistance in crop plants assume to be greater importance.

Considering the above facts, an attempt was made to investigate the genetic and functional diversity of fluorescent pseudomonads associated with green gram
rhizosphere and their consistent effect on alleviation of the water stress resistance with the following objectives:

1. Isolation, purification and preservation of fluorescent pseudomonads from rhizosphere soil of green gram (*Vigna radiata* L.) in Jorhat district of Assam (India).
2. Morphological and biochemical characterization of the isolated fluorescent pseudomonads.
3. DNA fingerprinting for analysis of genetic diversity of fluorescent pseudomonads isolates using various molecular markers.