Cocoa beans are the primary raw materials for confectioneries, beverages, chocolates and other edible products. The quality of beans is very important as cocoa components have antioxidant, antibacterial, antidiabetic, anticarcinogenic and cardioprotective properties. Cocoa has been found to be a suitable, profitable and important mixed crop in existing coconut (*Cocos nucifera* L.) and arecanut (*Areca catechu* L.) gardens in India. As a beverage crop it stands third after coffee and tea and as the most traded commodity in the world, it has second place after sugar. Based on 2012-2013 statistics, the major cocoa producing countries are Ivory Coast, Ghana, Indonesia, Brazil, Cameroon and Nigeria contributing 85% of the world total, whereas India’s contribution is negligible. The cocoa production capacity in India is inadequate to cater to the local market needs and every year there is an increased import of cocoa beans and its by-products. This situation offers a good scope for improving the productivity of this crop through various means. The application of inorganic fertilizers has had significant benefits on productivity in the short term. However, use of synthetic chemicals has raised a number of ecological problems in the last three decades. The current demand on residue-free produce has created a demand for the incorporation of biofertilizers in improving crop production. Microbial inoculants are promising components for integrated solutions to agro-environmental problems because they possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Mader *et al.*, 2011; Adesemoye *et al.*, 2009). Sustainable agriculture involves successful management of agricultural resources to satisfy human needs while maintaining environmental quality and conserving natural resources for the future. Currently there is considerable interest in exploiting the plant growth promoting rhizobacteria (PGPR) to improve crop production because of their rapid rhizosphere colonization and stimulation of plant growth.

Despite the acknowledged importance of soil and root-associated microorganism, little is known about the PGPR associated with *Theobroma cacao*. The present study paid attention on the evaluation of *Bacillus* spp. and fluorescent *Pseudomonas* spp. isolated from cocoa rhizosphere and roots as a first step toward a practical application of these microbes as agents of plant growth promotion. This study was focused on aerobic endospore-forming bacilli, particularly *Bacillus* spp.,
because of their tolerance to different environmental conditions and long-term viability in commercial preparations. And fluorescent pseudomonads, being a major group of rhizobacteria, which colonize aggressively the plant roots and are considered as an important group for sustainable agriculture. Isolation and evaluation of microorganisms associated with cocoa trees growing in different locations of South India, under different ecological conditions indicated that the fluorescent *Pseudomonas* spp. and *Bacillus* spp. occur in good numbers in this tropical plantation crop, corroborating the findings of Bopaiah and Shetty (1991), Melnick *et al.* (2011) and Lalitha Bai *et al.* (2011). Population density of *Bacillus* spp. was in higher number (up to 7.21 log cfu g⁻¹) than fluorescent *Pseudomonas* spp. (up to 4.24 log cfu g⁻¹) in rhizosphere soil and roots (up to 4.5 log cfu g⁻¹ *Bacillus* spp. and 2.4 log cfu g⁻¹ fluorescent *Pseudomonas* spp.). Similarly, higher population of *Bacillus* spp. (6.29 log cfu g⁻¹) and fluorescent *Pseudomonas* spp. (4.30 log cfu g⁻¹) in the rhizosphere soil of coconut, another important plantation crop in India was reported by Priya *et al.* (2011, 2012a). Melnick *et al.*, (2011) reported that the endophytic colonization of cocoa leaves by native bacilli varied by approximately 1.0 log cfu/cm². It is reported that these groups of bacteria exhibit multiple functional traits such as solubilization of inorganic phosphate and iron, production of vitamins, phytohormones and antimicrobial metabolites (Singh *et al.*, 2011a; Bhattacharyya and Jha, 2012). Population of fluorescent *Pseudomonas* spp. was lower in the roots of cocoa compared to rhizosphere soil and it ranges between 1.301 to 2.398 log cfu g⁻¹. The abundance of PGPR and their activities in the rhizosphere and roots are influenced by various environmental (e.g. soil type, nutrient status, pH, moisture) as well as plant factors (e.g. species, age, composition and pattern of root exudates). These results indicate that fluorescent pseudomonads and *Bacillus* spp. favour the association with cocoa and is home to diverse microbial community that is still only partially characterized. There was no significant variation in the population of *Bacillus* and fluorescent pseudomonads, under different agroclimatic conditions, which might be due to the comparable edaphic factors of the collected soil samples.

There is a need to isolate efficient microorganisms with a property of growth promotion, preferably from the same environment in which they are to be used. Such isolates will be more ecologically fit than the exotic strains. In search of efficient PGPR strains for improving the vigor of cocoa seedlings, 519 isolates were
collected from the rhizosphere soil and roots of cocoa. Out of these, 160 belonged to fluorescent *Pseudomonas* isolates (144 fluorescent *Pseudomonas* isolates from the rhizosphere and 16 root endophytic fluorescent *Pseudomonas* isolates) and 359 were *Bacillus* spp. (185 rhizospheric *Bacillus* isolates and 174 root endophytic *Bacillus* isolates). Amongst the PGPR, fluorescent pseudomonads and *Bacillus* spp. have received greater attention because of their ability to utilize a wide array of compounds as their carbon and energy sources and have diverse mechanisms for plant growth promotion (Cakmakci et al., 2010).

The selection of the most effective PGPR is an important prerequisite, in order to get maximum benefits from inoculated rhizobacteria (Hassen and Labuschagne, 2010), and therefore, two strategies were employed in the present study for screening the 519 isolates. The first strategy employed was the *in vitro* characterization of all isolates for different plant growth promotion traits (functional traits), which should be important to select PGPR with multiple PGP traits. Another approach was to conduct *in vitro* and *in vivo* tests, for evaluating the plant growth promoting potential on cowpea (short duration test crop for dicots). Similar screening approach was applied by Kumar et al. (2011), where they screened 80 isolates obtained from the rhizosphere of vegetable crops for antagonistic and PGP attributes to evaluate the genetic diversity. In 2007, Principe and coworkers (Principe et al., 2007) screened approximately 1,000 native strains isolated from saline soils of Cordoba Province of Argentina, to identify the strains which had PGP and biocontrol activities. Functional characterization of *Bacillus* and fluorescent *Pseudomonas* isolates suggested a considerable variation in growth promotion traits. *In vitro* tests such as production of IAA, ACC deaminase, HCN, siderophore, chitinases and antibiotics, ammonification, ability to grow on N-free medium and solubilization of tri-calcium phosphate revealed that 93% isolates could exhibit more than one PGP traits, which might promote plant growth directly or indirectly or synergistically. Multiple PGP activities among PGPR has been reported by many workers (Trivedi et al., 2011; Kumar et al., 2011; Chaiharn et al., 2009). PGPR with their multifunctional properties will attract more attention in the field of biofertilization. All the traits of this PGPR might not get expressed at a given point of time. Thus, the more the presence of the PGPR traits in an individual organism, the higher the chance of enhanced plant growth and yield (Abbas-Zadeh et al., 2010). In the current study, higher percentage of fluorescent
**Discussion**

*Pseudomonas* spp., exhibited PGP traits such as production of IAA, ACC deaminase, siderophore and phosphate solubilization whereas higher number of *Bacillus* spp. showed PGP traits like ammonification and ability to grow on N-free medium. Results of this study revealed that the cocoa rhizosphere and root endorhizosphere are inhabited by functionally diverse plant growth promoting *Bacillus* spp. and fluorescent *Pseudomonas* spp. with multiple activities. The present research will provide the basis for further understanding the functional properties of cocoa-associated bacterial community and open the possibility to manage the indigenous microorganism to optimize biological approaches for growth promotion of cocoa.

In the present study, 47% of isolates were able to solubilize insoluble tricalcium phosphate in Pikovskaya’s medium, indicating their potential role as a P-solubilizer. Percentage distribution of P-solubilizers were higher in fluorescent *Pseudomonas* isolates (60%) compared to *Bacillus* isolates (41%). Occurrence of high proportion of P-solubilizers associated with different crops was reported by many workers (Hayat et al., 2010). Deepa et al. (2010) reported that P-solubilizing soil bacteria could serve as efficient biofertilizer candidates for improving the P-nutrition of crop plants. Most phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants. The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can enhance the availability of phosphorus and iron for plant growth (Noori and Saud, 2012).

IAA production was found to be higher in fluorescent *Pseudomonas* isolates (75%) than in *Bacillus* isolates (32%). Both rhizosphere and endophytic *Pseudomonas* isolates showed 75% IAA production. It was reported that about 80% of bacteria isolated from plant rhizosphere are able to produce indole-3-acetic acid, one of the most extensively studied hormones which regulates cell division, cell elongation, cell differentiation and pattern formation in plants (Syed et al., 2010). Biosynthesis of IAA is not limited to higher plants. Organisms such as bacteria are able to make physiologically active IAA that may have pronounced effects on plant growth and development. Like in plants, L-tryptophan is considered as the IAA precursor in bacteria (Tsavkelova et al., 2007). Root exudates are natural source of L-tryptophan for rhizosphere microflora, which may enhance auxin biosynthesis in the rhizosphere. Production of IAA in the presence of a suitable precursor such as
tryptophan has been reported for several PGPR belonging to the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Pantoea*, *Pseudomonas* and *Serratia* (Syed et al., 2010; Arun et al., 2012; Priya et al., 2012b).

In the current study 199 isolates (96% fluorescent *Pseudomonas* and 13% *Bacillus* isolates) exhibited siderophore production, another important trait of the microorganisms that influences plant growth. Siderophore production by the isolate assumes significance for iron nutrition of plants grown under iron deficient conditions (Deepa et al., 2010) and for biocontrol (Peralta et al., 2012). There are several reports that showed microbial siderophores have a positive correlation with plant growth promotion and production of siderophores is one of the key factors that should be considered in PGPR screening programs (Sadegi et al., 2012; Peralta et al., 2012).

Majority of the tested isolates (75% fluorescent *Pseudomonas* and 91% *Bacillus* isolates) exhibited strong production of ammonia, which is taken up by plants as a source of nitrogen for their growth. Joseph et al. (2007) reported ammonia production in 95% of isolates of *Bacillus* followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45%). Accumulation of ammonia in soil may lead to increase in pH creating alkaline condition of the soil (pH 9–9.5). It suppresses the growth of certain fungi and nitrobacteria due to its potent inhibition effect (Jha et al., 2012). 70% of the isolates (out of 519) showed minimal growth in DF medium with ACC as the sole source of nitrogen. It has been postulated that sufficient build up of ACC deaminase possessing bacterial populations in plant root establish a sink for ACC, thereby lowering the endogeneous ethylene levels resulting in stress tolerance and enhanced root elongation (Jalili et al., 2009; Wu et al., 2012).

In the present study, only 61 isolates showed HCN production and less than 25% isolates produced antibiotics and chitinase enzyme. Cyanide acts as a general metabolic inhibitor to avoid predation or competition. The host plants are generally not harmfully affected by inoculation with HCN producing bacteria and host specific rhizobacteria can operate as biological control agents (Noori and Saud 2012; Saharan and Nehra 2011). Many PGPR are known to produce peptide antibiotics, which are oligopeptides that inhibit synthesis of the cell wall of pathogen, influence membrane structures of cells and inhibit the formation of initiation complex on small subunit of
Discussion

ribosomes (Maksimov et al., 2011). Bacterial antibiotics have an active influence in the regulation of the defense system of the plant. It was revealed that B. subtilis surfacine is able to stimulate induced systemic resistance by activation of components like lipoxygenases, lipid peroxidases and the formation of reactive oxygen species (Maksimov et al., 2011, Ongena et al., 2007). Applying bacteria which produce chitinases is effective for the biological protection of crops from pathogens, especially those that contain chitin and glucans within their cell wall structure (Maksimov et al., 2011). Present study suggests a high degree of functional diversity among fluorescent Pseudomonas and Bacillus isolates of cocoa. In 2012, Arun and co-workers (Arun et al., 2012) suggested that the wide differences in the ability to solubilize inorganic phosphates, production of IAA, ACC deaminase, salicylic acid, siderophore, HCN, antibiotics etc. among isolates from within and between the genera can be ascribed to their genetic makeup.

Based on the PGP criterion, a point scheme was generated to assess in vitro performance of the isolates and to select most promising candidates for further studies. Equal importance was given to all the PGP traits tested and a maximum of three points were given for each parameter totaling 27 points. Similar approach was applied by Furnkranz et al. (2009) when they screened 59 strains isolated from horseradish, sorghum, sunflower and safflower to identify the strains which had direct and indirect PGP traits. The rigorous screening of the 519 isolates for PGP attributes finally led to the selection of 104 promising bacterial isolates that were evaluated for plant growth promotion under in vitro conditions. Maximum score (14 out of 27) was acquired by 3 isolates (Fluorescent Pseudomonas sp. KGSF20, Bacillus sp. ASB12 and Bacillus sp. CSB17). These isolates could exhibit up to six PGP traits and they could solubilize phosphate, exhibit growth on N- free media and were capable of producing ACC deaminase, ammonia and siderophore in common. According to Barriuso et al. (2008), among the biochemical tests used to find putative PGP traits, these are the most common ones. In vitro screenings for bacterial PGP traits can provide a tool to select strains out of the vast numbers of bacteria living in plant associated habitats (Furnkranz et al., 2009). In 2011, Anuj and co-workers illustrated the significance of screening of rhizobacteria under in vitro conditions for multiple PGP traits and their evaluation in pot experiment. Hence, the selected 104 isolates were tested on cowpea (short duration test crop for dicots), for determining
Discussion

their plant growth promoting potential under *in vitro* (Plant growth chamber / seedling bioassay) conditions. Percentage increment in seedling length was higher in treatments inoculated with fluorescent *Pseudomonas* spp. KDSF7 and KDSF23. Fluorescent *Pseudomonas* sp. KDSF7 showed 100% increase and KDSF 23 showed 84% increase in seedling length of cowpea. Based on the selection criterion of concomitant increase in the tested parameters (i.e. shoot or root length) compared to uninoculated control, 88 isolates (including 30 fluorescent *Pseudomonas* isolates and 58 *Bacillus* isolates) were selected as promising ones. The good results obtained *in vitro* could not be dependably reproduced under *in vivo* conditions. The variability in the performance of PGPR might be due to various environmental factors that may affect the growth of the plant. Therefore, the selected isolates were further screened under greenhouse conditions in plastic pots containing unsterile soil. The results of our study under green house condition showed an increase in seedling length of cowpea (upto 28%), fresh weight (upto 52%) and dry weight (upto 38%) in response to inoculation with selected bacterial isolates. The percentage increase in dry weight of cowpea seedlings ranged between 2-38 and 1-28 in case of seedling length. The plant growth stimulation effect of the isolates tested can be explained based on their PGP traits. The incremented growth influence of the isolates in the test plant appeared promising for further testing as a bioinoculant for the cocoa plant. Hence, 21 PGPR (5 fluorescent *Pseudomonas* isolates and 16 *Bacillus* isolates) from a collection of 519 isolates were shortlisted based on their overall performance in the screening studies *viz.*, *in vitro* characterization for functional traits, seedling bioassay and green house studies. The chances of successful application of these isolates appeared high, as they were to be deployed in the same milieu from where they have been isolated.

In the current study, identification of the selected isolates were achieved by employing polyphasic methods. Phenotypical identification methods included the conventional biochemical tests and BIOLOG GEN III microbial identification system and the genotypical identification by 16S rDNA sequencing. The strains were tentatively identified based on colonial and cellular (light microscopic) morphology and conventional biochemical tests. Out of the selected 21 isolates, 5 were Gram-negative, non endospore-forming, motile rods, positive for catalase, oxidase, arginine dihydrolase and acid formation from glucose and xylose and they exhibited the characteristic fluorescence under UV light on King’s B medium. These characteristics
placed the isolates in the group fluorescent pseudomonads. Among these isolates *Pseudomonas* sp. KDSF7, which showed positive reaction for gelatin liquefaction, lecithinase reaction and growth at 41°C but negative for growth at 4°C was identified as *P. aeruginosa*. Four isolates (KDSF9, KDSF23, KGSF20 and KZSF6), were negative for gelatin liquefaction, lecithinase reaction and growth at 41°C but positive for growth at 4°C (except KZSF6) and they were identified as *P. putida*. Colony morphology of *Bacillus* isolates varied from round to irregular, flat, or raised, slimy to dry and medium sized to large opaque colonies. All isolates were Gram positive endospore-forming rods. Among the 16 *Bacillus* spp., 9 isolates (ASB12, CSB8, CSB16, CEB9, PEB2, PEB4, VEB4, VEB17 and KGE10) which were obligatory aerobes, positive for starch hydrolysis, nitrate reduction and citrate utilization, capable of tolerating 10% NaCl and exhibited good growth at 55°C were tentatively identified as *B. subtilis* isolates. Two *Bacillus* spp. (CSB17 and KGE16) showed analogous biochemical characters to *B. subtilis* isolates and differentiated on the basis of facultatively anaerobic growth were identified as *B. licheniformis*. Three facultatively anaerobic isolates (TSB15, KDSB3 and ASB3), exhibited positive reactions for Voges-Proskauer (V.P.) test, nitrate reduction, casein and gelatin hydrolysis, incapable of tolerating higher temperature and NaCl concentration were identified as *B. cereus* isolates. Obligatory aerobic, starch hydrolyzing, citrate utilizing and nitrate reducing *Bacillus* isolate WEB6 was identified as *B. megaterium*. *Bacillus* sp. TSB17, which showed similar biochemical characters to WEB6 except in case of variability in nitrate reduction, was tentatively identified as *B. kobensis*.

Identity obtained by conventional biochemical tests were validated based on their carbon substrate–utilization patterns obtained using the BIOLOG system. In the present study Biolog Gen III Micro Plates were used to test the ability of fluorescent *Pseudomonas* spp. and *Bacillus* spp. to metabolize major classes of biochemicals and physiological properties including, use of various sugars, amino acids, carboxylic acids, reducing power, pH, salt and lactic acid tolerance and chemical sensitivities. Biolog is a relatively simple and widely used method to assess the phenotypic/metabolic diversity of microorganisms (Priya et al., 2012b; Cakmakci et al. 2010). BIOLOG provided identification for 20 out of the 21 cocoa PGPR tested. Among fluorescent *Pseudomonas* spp., even though *Pseudomonas* spp. KGSF20, KDSF9 and KDSF23 were assigned as *P. putida* by conventional biochemical tests,
differential utilization of carbon sources such as D- mannose, D- fucose, inosine, D-serine, P-hydroxy phenylacetic acid, D-malic acid, tween 40, α-hydroxy butyric acid were found by BIOLOG GEN III microbial identification system. Among the 71 carbon sources 31 were utilized by P. putida KDSF9, 40 by P. putida KGSF20 and 30 by P. putida KDSF23. Out of the 23 chemical sensitivity assays 20, 19 and 18 were found to positive for P. putida KDSF9, P. putida KGSF20 and P. putida KDSF23 respectively. P. putida KDSF9 and KDSF23 were assigned as P. putida biovar B, P. putida KGSF20 as P. putida biovar A by BIOLOG GEN III identification system. P. putida KDSF9 and KDSF23 were assigned as P. putida biovar B, P. putida KGSF20 as P. putida biovar A by BIOLOG GEN III identification system. P. putida KDSF20 differ from other fluorescent Pseudomonas spp. in its ability to utilize α-hydroxy butyric acid as carbon source, to reduce tetrazolium blue and its resistance towards niaproof 4. Pseudomonas sp. KDSF7 differed from other fluorescent Pseudomonas spp. in phenotypic tests such as utilization of N-acetyl D-glucosamine, D- mannitol, gelatin, glycy1-1-proline, mucic acid, D- saccharic acid and was assigned as P. aeruginosa. Even though Pseudomonas sp. KZSF6 could utilize 57% of the tested C sources, it was identified only at the genus level.

Out of the 16 Bacillus spp., 10 isolates were identified as B. subtilis ss subtilis, one isolate was assigned as B. cereus/ thuringiensis, another as B. licheniformis, two as B. megaterium whereas one isolate could be identified only at the genus level. Even though 10 of the 16 Bacillus spp. were assigned as B. subtilis ss subtilis, there were large differences in the C source utilization. The discriminating substrates were stachylose, D- lactose, D-melibiose, N- acetyl mannosamine, N- acetyl D glucosamine, D- galactose, Methyl glucose, D-fucose, L-fucose, L- rhamnose, inosine, D- aspartic acid, Glycyl-1-proline, L-pyroglutamic acid, Lincomycin, guanidine HCl, glucuronic acid, mucic acid, quinic acid, D-sacharic acid, D-lactic acid methyl ester, ketoglutaric acid, D-malic acid, tween 40, G-amino butyric acid, acetoacetic acid, acetic acid, formic acid and aztreonam. None of the B. subtilis ss subtilis shared similar C-utilization profile. Two Bacillus spp. (TSB17 and WEB6), which were assigned as B. megaterium isolates, showed difference in the utilization of D-raffinose, D-glucose, L-alanine, L-aspartic acid, L-glutamic acid, L-pyroglutamic acid, D-gluconic acid, quinic acid, L- lactic acid, citric acid. B. subtilis ASB12 utilized higher number of carbon sources (66% of tested C sources) compared to the other strains. B. megaterium strains (WEB6 and TSB17) utilized very few number of carbon sources (18% and 17%, respectively). Only three substrates (N- acetyl D galactosamine, N-
acetyl neuraminic acid and L-galactonic acid) on the Biolog Gen III plates were not utilized by any of the tested isolates. None of the isolates was able to utilize 8 substrates out of the tested 94 carbon sources. Microplates contain some substrates that commonly occur in root exudates, which represent a primary source of carbon and energy and are likely to favor fast-growing microbes in the rhizosphere (Cakmakci et al., 2010; Alisi et al. 2005; Baudoin et al. 2003). The ability of selected isolates to utilize diverse carbon sources enable them to better compete with the natural microflora residing in the rhizosphere of plant hosts.

16S rRNA sequence analysis improves the identification of the isolates compared to phenotypic methods. In 2012, Ugur and co-workers (Ugur et al., 2012) suggested that identification with phenotypic methods should be verified by molecular methods in order to obtain meaningful conclusions. Hence, in the present study, 16s rRNA sequences of the selected PGPR were subjected to BLAST analysis. Isolates which were well identified at the species level by phenotypic method served as control for both conventional and molecular identification procedures. All the three methods were found in concord for identification of 11 of the 21 isolates but in some cases conflicting results were obtained. According to phenotypic characterization, KDSF9 was identified as *P. putida* while 16S rRNA sequence analysis showed maximum similarity (99%) with *P. plecoglossicida*. Even though both conventional biochemical analysis and Biolog GEN III identification suggested the identity of VEB4, CEB9 and CSB16 as *B. subtilis* and WEB6 as *B. megaterium*, 16S rRNA sequence analysis gave their identity only at the genus level. Both Biolog GEN III and 16S rRNA sequence analysis were found to be in agreement with TSB17’s identity (*B. megaterium*) whereas conventional biochemical analysis gave a different result. Several workers have described the necessity of 16S rDNA gene sequence analysis mainly to detect some misidentification among microorganisms (Ugar et al., 2012; Wu et al., 2006). Wherever 16S rRNA sequencing gave genus level identification, identity of isolates obtained with phenotypic methods were retained.

Digestion of the amplified 16S rDNA fragment with *Hae*III and *Hinf*I was the most discriminatory among the five restriction endonucleases for *Bacillus* and *Pseudomonas* isolates, respectively. In the present study, ARDRA analysis of *Bacillus* isolates using *Hhal* successfully differentiated *B. licheniformis* from *B. subtilis* whereas it could not distinguish *B. cereus* and *B. megaterium* isolates. Digestion using *Hae*III
could not distinguish \textit{B. licheniformis} from \textit{B. subtilis} but it gave different banding pattern for \textit{B. cereus} (except CSB17) and \textit{B. megaterium} isolates. ARDRA analysis of fluorescent \textit{Pseudomonas} isolates with \textit{Hin}fI produced separate banding patterns for \textit{P. putida}, \textit{P. plecoglossicida} and \textit{P. aeruginosa}. \textit{Hae}III could distinguish \textit{P. aeruginosa} from other fluorescent \textit{Pseudomonas} isolates.

Detailed studies on quantitative estimation for various PGP metabolites of selected PGPR showed that 17 of the 21 PGPR were able to solubilize tri-calcium phosphate within a range of 25.0 – 163.34 µg/ml, similar to those reported for phosphate solubilizing strains of \textit{Bacillus} spp. (Cakmakci \textit{et al.}, 2010; Naik \textit{et al.}, 2008). Maximum solubilization was observed with \textit{B. licheniformis} KGEB16 and the minimum solubilization with \textit{B. subtilis} PEB2. Significant decline in the pH of the culture medium by strains was observed with increased mineral phosphate solubilization, which suggested the microbial production of organic acids (Jha \textit{et al.}, 2012). Dey \textit{et al.} (2004), suggested that as the soil was deficient in available P, and soil pH was very conducive for P-solubilization, microbial P-solubilization would have played a role in better plant growth, yield and nutrient uptake. Several workers reported that phosphate-mobilizing microorganisms may be used as biofertilizers to increase P availability to plants, minimizing the use of expensive chemical P fertilizers (Singh \textit{et al.}, 2011a; Fernández, \textit{et al.}, 2012). Seven of the twenty one isolates produced IAA in \textit{in vitro} growth medium containing tryptophan. \textit{P. putida} KDSF23 and \textit{B. megaterium} WEB6 showed maximum IAA production (2.38 and 2.30 respectively) whereas other isolates produced IAA between 0.88 and 1.47 µg/ml. The critical role of IAA producing PGPR in plant growth promotion was documented by several workers (Jha \textit{et al.}, 2012; Hussain and Hasnain, 2011; Taghavi \textit{et al.}, 2010; Karnwal, 2009; Ahmad \textit{et al.}, 2008). It was reported that \textit{B. subtilis} strains secreting 0.7 µg/ml IAA increased rooting by 6.8% and root biomass in 106.7% in minicuttings of an \textit{E. grandis} x \textit{E. urophylla} hybrid (Teixeira \textit{et al.}, 2007). Fourteen isolates could produce salicylic acid ranging from 0.9 to 6.1 µg/ml. Chitinase and β - 1, 3- glucanase activities were recorded in 6 and 12 of the 21 PGPR, between 8.0 – 424.7 µg NAG/h/mg protein and 6.0 – 89.0 µg glu/min/mg protein respectively. These traits are considered to play an important role in plant defense responses against fungal pathogens (Saikia \textit{et al.}, 2011). Aeron \textit{et al.} (2011), found that the lytic enzymes such
Discussion

as chitinase and β-1,3-glucanase played a major role in suppressing the growth of *S. sclerotiorum* by degradation of the fungal cell wall.

The introduction and persistence ability of a strain are affected by a number of biotic factors like high salt, high water potential, high pH, and high temperature. The tolerance studies have highlighted the fact that our strains possess wide ecological tolerance properties. Test for the responses of the selected bacteria to different temperatures revealed that most of the *Bacillus* isolates (14 of the 16 *Bacillus* spp.) showed good growth at higher temperatures (≥ 50°C). Five *B. subtilis* isolates (CSB8, KGEB10, PEB2, PEB4 and VEB17) showed the potential to tolerate a maximum temperature of 60°C and were able to grow on TSA medium amended with 12% NaCl. *B. subtilis* CSB16, CEB9 and VEB4 were able to grow at 55°C and also showed intrinsic resistance to 12% of NaCl in TSA. The ability to adapt to temperature stress may be important in the survival of the microorganisms during drought. *B. cereus* CSB17, *B. licheniformis* KGEB16 and *B. subtilis* ASB12 could tolerate temperature of 55°C and bear NaCl concentration of 10%. The ability of the PGPR to adapt to high temperature and NaCl concentration in addition to the production of various PGP traits, certainly useful in order to formulate effective cultures which can survive and persist for longer period and work more efficiently under such climatic conditions. *B. cereus* KDSB3 and *B. cereus* TSB15 were the most sensitive strains to temperature (≤ 40°C). All the selected *Bacillus* spp. showed tolerance to 6% NaCl incorporated in TSA medium except one *B. cereus* KDSB3. *P. putida* isolates (KDSF23 and KGSF20) and *P. plecoglossicida* KDSF9 could tolerate low temperature up to 4°C. The growth of the test isolates under different pH condition was found to increase linearly from pH 5 to 7 and then slowly decline. Hard conditions in the field affect the expression of many microbial traits, application of PGPR strains that are tolerant to abiotic stresses such as temperature, salt, drought, oxidative stress and heavy metal toxicity is necessary (Sadeghi *et al*., 2012).

In the current study, effects of individual inoculation of selected PGPR (21 isolates) on cocoa seedling were evaluated for six months under field conditions in two separate trials. Results of the individual inoculation of selected PGPR showed that all the tested PGPR could increase at least one aspect of cocoa seedling growth. The growth parameters, namely root number (up to 38%), collar girth (up to 46%), number of leaves (up to 89%), total dry weight (up to 58%), total seedling length (up
to 37%) were increased significantly (P ≤ 0.05) due to PGPR inoculations. An incremental effect on growth was observed in cocoa seedlings on inoculation with PGPR reveals, broad spectrum plant growth promoting activity by the isolates. All the tested isolates possessed multiple functional traits and were able to utilize a wide range of carbon sources. This capability gives an added advantage (rhizosphere competence) to the inoculated strains compared to the ones which cannot utilize the source and showed less competence. In general, across treatments, it could be observed that all the tested PGPR enhanced collar girth (6- 41%), total seedling length (3-37%) and total dry weight (2- 58%) of cocoa seedlings compared with other growth parameters. Priya et al. (2012b) reported that girth in the collar region is an important character which determines the seedling vigour. Enhancement of seedling length and dry weight observed with bacterization were documented earlier (Brahmaprakash and Pramod Kumar, 2012; Jha et al., 2012; Erturk et al., 2010). The lack of phytopathogenic symptoms in the uninoculated control indicated that there were no such challenges to the seedlings during the experiment. Direct action was, therefore, the likely mode used by the isolates to enhance the growth of cocoa seedlings. PGPR treated seedlings also maintained significantly (P≤0.05) higher beneficial microbial populations in the rhizosphere of cocoa seedlings corroborating the findings of Guo et al. (2011). He observed that the bacterial population in the rhizosphere soils of S. alfredii inoculated with Burkholderia sp. D54 increased by 54% compared with the uninoculated control. Root exudates (carbohydrates, amino acids, organic acids) and mucilage-derived nutrients attract deleterious rhizobacteria as well as beneficial and neutral bacteria allowing them to colonize and multiply in the rhizosphere. In inorganic farming a modification of natural balance can drastically alter the microbial community leading to the loss of beneficial microbes and ingress of plant pathogens, which may have a devastating effect on plant productivity (Avis et al., 2008). Such detrimental soil microbial changes in cocoa plantation can be prevented by use of these PGPR.

Cocoa seedlings bacterized with three fluorescent Pseudomonas isolates, P. putida KDSF23, P. aeruginosa KDSF7 and P. putida KGSF20 resulted in the highest mean total seedling length (160.6 cm, 160.1 cm and 159.9 cm respectively) compared to other treatments in the first trial. Inoculation of P. putida KDSF23 and P. aeruginosa KDSF7 exerted greater influence in increasing the total dry weight of seedlings.
These effects may be due to the high colonization ability of fluorescent Pseudomonas spp. which helped in the rapid establishment in the rhizosphere of cocoa seedlings along with their beneficial PGP traits. However, another fluorescent pseudomonad isolate P. putida KZSF6, was grouped among one of the least performing treatment. The differences in plant growth promotion among these isolates are attributed to the difference in their mode of action and individual rhizospheric competencies, since the study was taken in unsterile soil. B. licheniformis KGEB16, B. subtilis ASB12, B. subtilis PEB2 and B. cereus CSB17 were found to be the prominent growth promoters among Bacillus spp. in the first trial. In vitro studies showed that these PGPR were able to tolerate high temperature (55°C) and NaCl concentration (10%) and exhibited multiple PGP traits. Studies on the patterns of carbon source utilization by these PGPR revealed that all the four isolates could utilize a broad range of carbon sources (more than 60% of tested C sources). The phytostimulatory effect of these isolates on cocoa seedlings may also attribute on these beneficial characteristics because the least performing Bacillus isolates (B. cereus KDSB3, B. cereus TSB15 and B. megaterium TSB17) could utilize less number of C sources (32%, 39% and 17% of tested C sources) in the BIOLOG GEN III microplate (substrates that commonly occur in root exudates). The ability to utilize wide range of compounds will provide a selective advantage to the strains that uses the substrate and may lead to the dominance of inoculated PGPR, and could have a high potential for use as a biofertilizer in agriculture. Comtant et al. (2010) suggested that the ability of PGPR to utilize diverse carbon and energy sources enable them to be highly competitive to successfully colonize the root zone. In the first trial, maximum enhancement in the different growth parameters such as number of leaves (33%), total seedling length (16%), total nitrogen content in the rhizosphere soil and leaves of cocoa seedlings were recorded with inoculation of P. putida KDSF23, clearly showing the beneficial role of this rhizobacteria. The growth promotion efficacy might be attributed to the production of IAA, siderophore, ACC-deaminase, salicylic acid, chitinase and β-1-3 glucanase by P. putida KDSF23 and any other PGP activity of this isolate in favor of plant growth response. The favorable effects of these PGP traits on plant growth have been previously reported (Bhattacharya and Jha, 2012). Earlier reports revealed that low level of IAA produced by PGPB promotes plant growth, in our study the value of IAA produced by P. putida KDSF23 (2.38 µg/ml) may be within the reasonable range influencing the growth. Regulation of ethylene level and promotion of plant growth
Discussion

by ACC-deaminase containing bacteria has been documented (Wang et al., 2012). Syed et al. (2010) suggested that the bacterium could be indirectly augmenting the availability of phosphorus because siderophore production is one of the mechanisms involved in the solubilization of iron-bound phosphorus by the microorganisms. Available P in the rhizosphere soil and total P content in the leaves were highest with the bacterization of B. licheniformis KGEB 16 compared with other treatments. The same isolate exhibited maximum P- solubilization (163.34 µg/ml) under in vitro analysis. The strong ability of the strains to solubilize inorganic phosphate may have much influence on enhancing the nutrient content, which is in agreement with the result reported by Guo et al., 2011. Noori and Saud (2012) reported that PGPR could solubilize precipitated phosphates and enhance phosphate availability to plant, it represent a possible mechanism of plant growth promotion under field conditions. Phosphate solubilizing bacteria are also known to increase phosphorus uptake resulting in better growth and higher yield of different crop plants (Cakmakci et al., 2010; Verma et al., 2010).

In the second trial B. cereus ASB3 and B. subtilis VEB4 were found to be the best plant growth promoter in cocoa seedlings. These isolates showed significant (P≤0.05) increase in all the tested growth parameters. Maximum enhancement in the total dry mass (56%), phosphorous content in leaves and nitrogen content in the rhizosphere soil of cocoa seedlings were observed in the inoculation of B. cereus ASB3. Cocoa seedlings bacterized with B. subtilis VEB4 showed maximum enhancement in total seedling length (37%), collar girth (27%) and total nitrogen content of leaves. Earlier studies on in vitro analysis of PGP traits revealed that B. cereus ASB3 had an intrinsic ability for the solubilization of phosphate, production of ACC deaminase while B. subtilis VEB4 had the ability to grow well on N free medium and was a good ammonifier. The possible mechanism involved behind these beneficial effects is the presence of these plant growth promotion traits. These isolates might have helped in better solubilization of P and fixation of nitrogen as revealed from significantly higher nutrient content in soil and leaves. All the treatments showed enhancement in the number and length of leaves, number of roots, collar girth, total seedling length, total fresh and dry weight of cocoa seedlings compared to control in the second trial. The capability of utilizing wide range of carbon sources by these PGPR except B. megaterium WEB6, as revealed from BIOLOG
studies may have helped them to dominate in the rhizosphere region and exert their phytostimulatory effect. Significant increase in the number, length and dry weight of the roots of cocoa seedlings inoculated with *B. megaterium* WEB6 might have been due to their ability to produce IAA and ACC deaminase. All the treatments in the second trial, also maintained a good number of P-solubilizers in the rhizosphere soil of cocoa seedlings compared to untreated control. Influence of PGPR on the growth, development and yield of different crops under controlled and varied natural conditions either directly or indirectly, following various mechanisms were documented earlier (Almoneafy *et al*., 2012; Singh *et al*., 2011b; Kumar *et al*., 2011). The high level of positive responses with respect to total seedling length, dry weight of shoot and root and collar girth of cocoa seedlings, parameters that indicate seedling growth, were observed with application of *P. putida* KDSF23, *B. licheniformis* KGEB16, *B. cereus* ASB3 and *B. subtilis* VEB4 in individual inoculation studies.

Reports suggest that application of binary or multiple mixtures would mimic the natural situation more closely and might broaden the spectrum of growth promotion (Raupach and Kloepper, 1998). Hence a combination (dual) of most promising isolates, selected based on individual inoculation studies (*B. cereus* ASB3, *B. subtilis* VEB4, *B. licheniformis* KGEB16 and *P. putida* KDSF23) were evaluated on cocoa seedlings in polybags under field conditions. The *in vitro* compatibility studies revealed that these PGPR did not show any inhibitory effect with each other. According to the growth parameters considered, significant differences were found between the individual and dual inoculations of PGPR. Combined treatments involving *P. putida* KDSF23 had no further stimulatory effect on the growth parameters of cocoa seedlings, which perhaps indicates a competition effect although they were compatible under *in vitro* conditions. This can be attributed to differential behavior of isolates to composition of root exudates, temperature variation or to their interaction with rhizosphere microflora. Perhaps the coexistence of both strains (without competition) might have increased the quantity of one PGPR, and thus, the concentration of metabolites involved in growth promotion exceeded the optimum concentration for a positive effect on plants or both strains produced a decrease in metabolite synthesis, which obviously affect the plant growth. Dual inoculation of *B. licheniformis* KGEB16 with *P. putida* KDSF23 and *B. cereus* ASB3 resulted in significant positive increase in number of roots, root length, total seedling length and total N
content in leaves compared with \textit{B. licheniformis} KGEB16 individual inoculation. Synergistic effect in the co-inoculation of \textit{B. licheniformis} KGEB16 with \textit{P. putida} KDSF23 could be attributed to the ability of these isolates to synthesize indole acetic acid, which may give cumulative effect to modify the root architecture and thereby increase uptake of major nutrients. Combination of P-solubilizing \textit{B. licheniformis} KGEB16 with another P- solubilizing \textit{B. cereus} ASB3 might have resulted in additive effects on P uptake as evidenced by the high P content in the leaves. Total P and K content in leaves, total N content and available K in rhizosphere soil were found to be on par with individual inoculation of \textit{B. licheniformis} KGEB16 and dual inoculation of \textit{B. licheniformis} KGEB16 + \textit{P. putida} KDSF23. Co-inoculation of \textit{B. licheniformis} KGEB16 + \textit{B. subtilis} VEB4 exhibited a greater phytostimulatory effect on cocoa seedlings than its individual inoculations. Dual inoculation of \textit{B. subtilis} VEB4 + \textit{B. licheniformis} KGEB16 showed 29\% increase over \textit{B. subtilis} VEB4 and 14\% over \textit{B. licheniformis} KGEB16 in case of total dry weight. The highest total seedling length of 176.7 cm and significant positive effect in the total N, P and K content in leaves, were recorded in treatments which received combined inoculation of \textit{B. subtilis} VEB4 + \textit{B. licheniformis} KGEB16 compared to their individual inoculations. The enhanced growth may be attributed to the auxin production by \textit{B. licheniformis} KGEB 16, which is known to stimulate root growth, thereby facilitating an increased uptake of nutrients from the soil. An earlier observation by Khalid \textit{et al.} (2004), observed a linear positive correlation between \textit{in vitro} auxin production and plant growth promotion by rhizobacteria which corroborates our results. Another possible factor is the enhanced availability of soluble P in the rhizosphere region, as a result of the P solubilization by the bacterium. This treatment also maintained a good population of \textit{Bacillus} spp. and P-solubilizers in the rhizosphere soil of cocoa seedlings. The presence of high number of bacteria in the rhizosphere is undoubtedly also important, since they may convert organic and inorganic substances into available plant nutrients. Combined inoculation of \textit{B. subtilis} VEB4 with other PGPR, also performed better in cocoa seedlings than its individual inoculation. The increase in the growth parameters of cocoa seedlings by combined inoculation of PGPR could possibly be due to the cumulative effects, such as enhanced supply of NPK to the crops in addition to growth promoting substances produced by these organisms. The increase in growth of cocoa seedlings could also be due to nutrient supplementation among the inoculated organisms, which might have enhanced their efficiencies. The
ability to utilize broad range of C sources and differences in the C source utilization pattern among these PGPR as revealed from BIOLOG studies will help them to coexist in the rhizosphere region without much competition and exert their phytostimulatory effects. Dey *et al.* (2004) suggested that the capability of utilizing wide range of carbon and energy sources by bacteria is essential in the initial stages of multiplication and establishment in the rhizosphere. Previous studies on the co-inoculation of PGPR revealed that most of the microorganisms used in mixers improved the absorption of nitrogen, phosphorus and mineral nutrients by plants (Figueiredo *et al.*, 2010; Yadegari *et al.*, 2010). Combined inoculation of *Rhizobium* with *Pseudomonas striata* or *B. megaterium* led to increased dry matter, grain yield and phosphorus uptake significantly over the uninoculated control in legumes (Elkoca *et al.*, 2008). Verma *et al.* (2010) reported the positive influence of *Rhizobium* spp. and plant growth promoting rhizobacteria on nodulation, plant biomass and yields of chickpea plants. Co-inoculation of *Pseudomonas* spp. with *Rhizobium* improves growth and symbiotic performance of fodder galega (Egamberdieva *et al.*, 2010). Yadegari and Rahmani (2010) showed that co-inoculation of three *Phaseouls vulgaris* cultivars with two *Rhizobium* strains, *P. fluorescens* and *A. lipoferum*, resulted in increased seed yield, number of pods per plant, weight of seeds, seeds protein yield and number of seeds per pod. Such effective combinations should reduce the use of hazardous chemicals and their adverse effects on ecosystems. It is thus reasonable to suggest that manipulation of the composition of microorganisms in the rhizosphere with sustainable effects could be best accomplished by introducing mixtures of compatible microorganisms.

It has been indicated that less than 50% of chemical fertilizers is absorbed by plants and the rest would not be accessible to the plant as it is subjected to leaching, runoff, and emission from the soil surface (Adesemoye *et al.*, 2009). Hence, the use of biological fertilizers as supplementary fertilization to chemical fertilization is necessary. Soil microbes are a big help to plant and the environment as they own some great abilities that collectively enhance plant growth. In the presence of soil microbes, plant absorbs higher amounts of nutrients and less risk of environmental pollution is likely (Miransari, 2011). Methods for inoculation with PGPR require the use of a carrier to deliver the inoculum into the soil and allow mixing of the cells in the soil profile. In fact, formulations that transfer the growth promoting activities of a
strain from laboratory to field would have a major impact in agriculture. Vidhyasekaran et al. (1997) used powder formulations of *P. fluorescens* to control pigeonpea wilt effectively. Hence in this study talc based formulations of the selected PGPR were prepared and shelf life was assessed for six months. The result indicated that the shelf life of PGPR in talc formulation varied with the isolates during the storage period. The population of *B. cereus* ASB3, *B. licheniformis* KGB16 and *B. subtilis* VEB4 in talc formulations was retained 8.46, 8.29 and 8.38 log cfu g\(^{-1}\) respectively, even after 6 months of storage, which corroborated the findings of Vidhyasekaran and co-workers (Vidhyasekaran et al., 1997). These endospore forming *Bacillus* isolates can persist in storage from months to years, and can withstand starvation, temperature, moisture and other environmental stresses better than nonspore forming bacteria. *P. putida* KDSF23 could maintain a population of 7.22 log cfu g\(^{-1}\) after 180 days of storage. The population of the isolate in the formulation declined later on. Several workers demonstrated the potentiality of talc to be used as a carrier for formulating rhizobacteria (Ardakani et al., 2010; Sah et al., 2011; Chakraborty et al., 2009; Rajendran and Samiyappan, 2008). Aeron et al. (2011) reported that the stability of *P. fluorescens* PS1 was maintained even after 360 days of storage in sawdust + soil formulation. Shelf life studies using talc based powder formulation of potent PGPR demonstrated that it can maintain a higher population of PGPR (10\(^7\) to 10\(^8\) cfu g\(^{-1}\)) at room temperature after 180 days of storage than the recommended population (10\(^7\) cfu g\(^{-1}\)) as per Bureau of Indian Standard (BIS)(Specification for Inoculants, 2002). Present study suggests that the inoculated microorganisms can be retained in soil and biopriming with these PGPR could help increase the vigour of cocoa seedlings and reduce the amount of fertilizer input by increasing efficiency of nutrient availability/uptake. Functionally diverse plant growth promoting bacteria, which can utilize diverse carbon sources brought forward from the current study, can be used in organic farming as natural bioresources that would not only enhance agricultural productivity but also maintain soil quality.