CHAPTER 5
DISCUSSION

5. DISCUSSION

Manipur, one of the states in North Eastern Region of India is well known for its richest reservoir of plant diversity and considered as one of the 'biodiversity hotspot' region of India. In the present study, *Achyranthes aspera* L. growing in Manipur was selected because of its known medicinally properties (Dey, 2011). Instruction on role of endophytic bacteria of *A. aspera* for its growth promotion and effect on anti-oxidant activity of *A. aspera* is very limited. Though role of endophytes in mediating the anti oxidant property of the plant is reported from other plants that improve the medicinal properties of the plant (Hamilton *et al*., 2012). Endophytes have been defined as 'microbes that settle living, interior tissues of plants without causing any instant, exert negative effects' (Bacon and White, 2000). Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species (Ryan *et al*., 2008). Currently, endophytic bacteria associated with medicinal plants have attracted the attention of several investigators. Isolation of endophytic *Serratia marcescens* from different plants such as edible cactus, summer squash, rice were already reported (Li *et al*., 2011; Selvakumar *et al*., 2007; Gyaneshwar *et al*., 2001). Isolation of *S. marcescens* from the rhizoplane of *A. aspera* L for phosphate solubilization has been already reported (Misra *et al*., 2012). *S. marcescens* is being reported from *A. aspera* leaves, which also have the plant growth promoting activities. Isolation of endophytic *Pseudomonas aeruginosa* PM389 has been reported from several plants including pearl millet, however *P. aeruginosa* AL2-14B is reported from *A. aspera* leaves. *Bacillus methylotrophicus* have been reported as an endophyte, from root endosphere of healthy apple, while *B. methylotrophicus* from *A. aspera* stem has not been reported previously. This study

demonstrates the effectiveness of endophytic bacteria isolated from *A. aspera* L. containing PGPB traits which improve the growth of the plant. The growth of *A. aspera* inoculated with potential bacteria was tested by pot trial experiment and its effect on the antioxidant property of the plant was also determined.

In the present study, *P. aeruginosa* AL2-14B, *S. marcescens* AL2-16, *S. marcescens* AL6-10 and *B. methylotrophicus* AST5-2 were capable of producing IAA with or without the supplement of L-tryptophan. Misra *et al*. (2012) reported the production of IAA by *S. marcescens* isolated from the rhizoplane of *A. aspera* which produced 65.5 µg/ml. Our results showed that the endophytic *S. marcescens* AL2-16 and AL6-10 isolated from the leaves of *A. aspera* L. were found to release appreciable amount of IAA i.e. 133.2 µg/ml and 107.56 µg/ml respectively which was induced by 1.0% L-tryptophan, suggesting that these isolates has tryptophan dependant IAA release mechanism. Maximum IAA was released after 144 h and 96 h of incubation by *S. marcescens* AL2-16 and AL6-10 respectively. These findings are consistent with the previous observation that *S. marcescens* strain RSC-14 synthesize IAA which was confirmed by using Salskowski assay and a gas chromatography mass spectrometric analysis (Khan *et al*., 2017). Selvakumar *et al*. (2008) reported that the amount of IAA production by endophytic *S. marcescens* strain SRM was 11.1 µg/ml after 48 h of incubation with the addition of 100 µg/ml of L-tryptophan concentration. Gupta et al. (2012) reported that *S. marcescens* A51 produced 28.2 µg/ml of IAA. Jha and Kumar (2007) also reported the presence of IAA in the culture filtrate of endophytes from *Typha australis* in which seven of 10 endophytic isolates were positive for IAA production. All the 18 isolates of endophytic bacteria isolated from roots and stems of tomato

(*Lycopersicon esculentum)* plant which was collected from different regions of Gujarat exhibited a significant amount of IAA production (Patel *et al*., 2012). Liu *et al*. (2010) reported that *Serratia* sp. G3 isolated from the stem of wheat has the ability to produced the plant growth regulator indole-3-acetic acid. *P. aeruginosa* AL2-14B also produced IAA (114.79 µg/ml) which was in agreement with earlier reports (Khamna *et al*. 2010; Sachdev *et al*. 2009). Kumar *et al*. (2016) reported that *P. putida* ECL5 produced 23 µg/ml of IAA on supplementation of 400µg/ml of L-tryptophan after 48 h of incubation. Fouzia *et al*. (2015) reported that *P. fluorescens* CHAO, *P. fluorescens* RB13 and *P. aeruginosa* EH4 produced 88.37µg/ml, 50.95µg/ml and 36.88 µg/ml of IAA after 48 h of incubation respectively, with supplement of 5 µg/ml of L-tryptophan. Khamna *et al*. (2010) reported that the amount of IAA production by *Streptomyces* CMU-H009 was 143.95 µg/ml after 3 days of incubation with the addition of 2 mg/ml of L-tryptophan concentration. *Klebsiella* strain K8 produced 22.7 mg/l of IAA under optimum conditions at 72 h of incubation (Sachdev *et al*., 2009). Akbari *et al*. (2007) reported that *Azospirillum* strain 118-I produced 285.51 mg/l of IAA. *Bacillus methylotrophicus* AST5-2 also produced 102.26 µg/ml of IAA with the addition of 0.6% L-tryptophan after 48 h of incubation. This result was supported by Khan *et al*. (2016) where endophytic bacteria *Bacillus* sp. LK14 isolated from bark of *Moringa peregrine* showed significantly higher IAA production (8.7 µM) on 14th day of growth by spectrophotometry analysis method. Li *et al*. (2016) found out that *Bacillus* sp. pp02 isolated from the roots of elephant grass had the ability to produce IAA at a range 10.50-759.19 mg/l. Junior *et al*. (2015) reported that *Bacillus* sp. UFTBt07, *Bacillus* sp. UFTBc19, *Bacillus* sp. UFTBc produced 1253 µg/ml, 1043 µg/ml, 990 µg/ml of IAA respectively with the addition of

100 mg/l of L-tryptophan after 96 h of incubation. The property of synthesizing indole acetic acid is considered as an effective tool for selecting favourable microorganisms as there have been reports suggesting that IAA producing bacteria have reflective effect on plant growth (Kloepper *et al*., 1980, Kuklinsky-Sobral *et al*., 2004, Govindarajan *et al*., 2007).

Siderophores play an essential role in the microbial interactions, enhancing the growth of plants and yield of agricultural crops. *P. aeruginosa* AL2-14B, *S. marcensens* AL2-16 and S. *marcensens* AL6-10 were found to produce siderophore in good amounts as evident by CAS assay whereas *B. methylotrophicus* AST5-2 gave negative result. In fact, 71.806% unit of siderophore was recorded in succinate broth after 72 hours of incubation by *P. aeruginosa* AL2-14B. The pattern of siderophore release was correlated with growth, and results were in accordance with similar study on siderophore production reported in *Azospirillurn* (Saxena *et al*. 1986), where maximum siderophore production was recorded after 20 h of growth. Pyoverdine type siderophore in *P. aeruginosa* PAO1 was also recorded to be released in maximum amount after 40 h of growth (Barbhaiya *et al*. 1985). *Pseudomonas* sp. G-229-21 could produce high-affinity carboxylate type siderophores under low iron conditions and its siderophores were capable of antagonising against *Phytophthora parasitica* var. *nicotianae* (Breda de Hann) Tucker strongly under low iron conditions (Tian *et al*., 2008). In earlier reports, endophytic *P. aeruginosa* PM389 isolated from the healthy pearl millet plant produced catecholate type of siderophore (63.5% units) which exhibited siderophore index of 0.711 (Gupta *et al*. 2013). Pandey *et al*. (2005) reported that *P. aeruginosa* GRC1, isolated from mustard plant, produced 18.76 µg/ml of hydroxamate type of siderophore at the iron concentration

of 0.2µM. Whereas *S. marcescens* AL2-16 and *S. marcescens* AL6-10 produced 74.931% and 81.188% of siderophore units after 48 h of incubation. This result was supported by Khilyas *et al*. (2016) where two strains of *S. marcescens* SM6 and SR41-8000 started to produced siderophore after 12 h of incubation, reached its maximum at 30 h of growth and remain constant on further incubation. Gupta *et al*. (2012) reported that *S. marcensens* A51 produced 86.67% of siderophore units. Afzal *et al*. (2015) also reported that endophytic *S. marcescens* MOSEL-W2, isolated from *Cannabis sativa* produce siderophore. Seyedsayamdost *et al*. (2012) reported that a novel *Serratia* sp. designated as *Serratia* sp. v4 that produced serratiochelin and an analog of serratiochelin. Serratiochelins are bis-catecholate siderophore and may be tetra or hexadentate.

In all these cases, the maximum siderophore concentration in the culture supernatant was seen at a time when the culture just entered late log phase and siderophore production occurred parallel with growth. The most suitable medium for the siderophore production was found to be Succinic Medium since it gave maximum yield of siderophores. This was similar from the earlier report where *P. fluorescens* NCIM 5096 produced 72% of siderophore unit in Succinic medium (Sayyed *et al*. 2005). Among carbon source, fructose resulted in the maximum siderophore production. It is known that iron starvation stimulates the production of siderophores. Siderophore release was induced in a very low amount of iron. A gradual decrease in siderophore production thereafter, with an increase in iron concentration suggested that the siderophore production by *P. aeruginosa* AL2-14B, S. *marcescens* AL2-16 and S. *marcescens* AL6-10 are under strict control of iron concentration. The most suitable iron concentration for siderophore production unit was 1 µM for both *P. aeruginosa* AL2-14 B and S.

marcescens AL2-16. Sayyed *et al*. (2005) also found maximum siderophore production at 1µM of iron by *P. fluorescens* NCIM 5096 and *P. putida* NCIM2847.

It is known that bacteria can solubilize inorganic phosphate because of their ability to produce and release organic acids, which through their carboxylic groups chelate the cations (mainly Ca) bound to phosphate converting them into the soluble forms (Kpomblekou *et al*. 1994). The isolates *P. aeruginosa* AL2-14B, *S. marcescens* AL2-16, *S. marcescens* AL6-10 and *B. methylotrophicus* AST5-2 were able to formed halo zone when inoculated on the Pikovskaya's solid medium containing tri-calcium phosphate. The pH of the medium decreased with the increase in the amount of free phosphate released, showing maximum P solubilization at pH 3.9 after 144 h of incubation (383 µg/ml) by *P.aeruginosa* AL2-14B. Similar results were reported earlier by Ahmed and Khan (2010) indicating that *P. aeruginosa* PS1 can solubilize 385 µg/ml of inorganic phosphate after 168 h of incubation. Kaur *et al*. (2013) reported that *Pantoeacypripedii* PSB-3 and *Pseudomonas plecoglossicida* PSB-5 isolated from the rhizospheric soil of *Stevia rebaudiana* can solubilize 253 mg/ml and 271 mg/ml of inorganic phosphate respectively after 5 days of incubation. Paul and Sinha (2017) revealed that *P. aeruginosa* KUPSB12 has phosphate solubilization index of 2.85 in Pikovskaya's agar plates along with high soluble phosphate production of 219.64±0.330 µg/ml in liquid medium. Oteino *et al*. (2015) reported that *Pseudomonas* sp. L228, *Pseudomonas* sp. 132 and *Pseudomonas* sp. L111 can solubilized 1312 µg/ml, 1024 µg/ml and 438 µg/ml respectively. Park *et al*. (2010) reported that *Pantoea* sp. CS2-B1 and *Enterobacter* sp. SM1-B1 isolated from phosphate-amended and lead contaminated soils solubilized 475mg/l and 293 mg/l of phosphate, respectively. The isolates *S. marcescens* AL2-16 and

S. marcescens AL6-10 were also formed halo zone when inoculated on the Pikovskaya's solid medium containing tri-calcium phosphate. Both the isolates *S. marcescens* AL2-16 and *S. marcescens* AL6-10 produced maximum P solubilization at pH 4.9 (259 µg/ml) and pH 4.3 (353 µg/ml) after 72 h of incubation respectively and result was supported by George *et al*. (2013) where he reported that *S. marcescens* KiSII isolated from the rhizospheric soil of coconut can solubilized 216 µg/ml of inorganic phosphate after 72 h of incubation. Behera *et al*. (2017) reported that *Serratia* sp. PSB-37 exhibited maximum phosphate solubilization of 44.84 µg/ml at 48 h of incubation with a maximum drop in the pH of 3.15 of the medium. Gupta *et al*. (2012) also reported that *S. marcescens* A51 can solubilized 212 µg/ml of inorganic phosphate. *S. marcescens* NBRI1213 showed maximum phosphate solubilization activity of 984 µg/ml (Lavania and Nautiyal, 2013). *B. methylotrophicus* AST5-2 can also released 149 µg/ml of phosphate at pH 5.2 after 48 h of incubation. Results of present work was supported by Oteino *et al*. (2015) where *Bacillus* sp. S20 showed phosphate solubilization activity of 85 µg/ml of phosphate at pH 5.28. Panda *et al*. (2015) reported that *Bacillus* sp.M510, *Bacillus* sp. R42, *Bacillus* sp. R81 has the ability to solubilized tricalcium phosphate with the solubilization rate of 158 µg/ml, 115 µg/ml, 34.6 µg/ml respectively after 12 days of incubation. The *Bacillus* sp. NPSBS3.2.2 which was having less phosphorous solubilizing efficiency from primary screening (25%) solubilized maximum amount of Phosphorous in Pikovskaya's broth (0.129¼g/ml) (Narveer *et al*., 2014). Nautiyal (1999) reported that *Bacillus* sp.1 can solubilized 17 µg/ml of phosphate in Pikovskaya's broth.

Nitrogen is the most important limiting factor for plant growth in various environmental conditions. Therefore, sufficient utilizable form of nitrogen must be

provided to the plants for optimum growth and yield. Biological nitrogen fixation is considered to be the most potential way to provide the fixed form of nitrogen to the plants. Most of the biologically fixed nitrogen made available to the plants is contributed by *Rhizobium* sp. and cyanobacteria, which form association with specific plant species. It restricts their use in agriculture to certain plants only. Discovery and characterization of other associative and endophytic diazotrophic bacteria obtained from diverse plant species in the last couple of decades have raised prospects of their use as biofertilizer (Bhattacharjee *et al.,* 2008). Application of endophytic bacteria as an effective agent for sufficing nitrogen requirement and other plant growth promoting properties to associated host plant appears to be one of the most promising approaches in the sustainable agriculture system. Nitrogen fixing ability of bacteria was screened by growing them on N-free medium. This was further confirmed by *nifH* gene amplification. Quantitative estimation of fixed nitrogen was done by ARA (Acetylene reduction assay). Amplification of *nifH*, *nifD*, and *nifK* by PCR or RT-PCR has been frequently employed in detection of N_2-fixing ability of bacterial and cyanobacterial isolates (Chowdhury *et al*., 2007; Bothe *et al*., 2010). DNA sequence of *nifH* of different diazotrophic bacterial species has been reported to be one of the most conserved sequences which can be used for identification of species (Izquierdo and Nusslein, 2006). Therefore, gene sequence of *nifH* is used for probing of nitrogenase among diazotrophic bacteria as well as analysis of diazotrophic communities growing in diverse environmental conditions (Jha and Kumar 2009; Bothe *et al*., 2010). All the four selected isolates viz, *P. aeruginosa* AL2-14B, *S. marcescens* AL2-16, *S. marcescens* AL6-10 and *B. methylotrophicus* AST5-2 grew in plates containing nitrogen-free medium. This was further confirmed by Acetylene

reduction assay (ARA) by using Gas chromatography-Flame Ionization Detector (GC-FID). The desired amplicon of 781 bp corresponding to *nifH* gene was obtained by *P. aeruginosa* AL2-14B, *S. marcescens* AL2-16 and *S. marcescens* AL6-10. Nitrogenase activity was quantified and it was found to be 1.8617 nmol ethylene μg^{-1} $protein^{-1}$ hr^{-1} by *P. aeruginosa* AL2-14B as detected using GC-FID technique, which is an important attribute for endophytic PGPR. N-fixing endophytic bacteria are better than their rhizospheric and rhizoplanic counterparts as they provide fixed nitrogen directly to their host (Cocking, 2003). Moreover, endophytic bacteria are less vulnerable to competition with other soil microbes for scarce resources and remain protected to various abiotic and biotic stresses (Reinhold-Hurek and Hurek, 1998). Earlier, Taule *et al*. (2012) and Reinhard *et al*. (2008) had reported N-fixing endophytic *Pseudomonas* sp. from sugarcane and maize plants respectively. Yan *et al*. (2008) reveal that *Pseudomonas* sp. A1501 has the ability to fixed atmospheric nitrogen because of the presence of *nif* genes. Similar result was also reported by Kathiravan and Krishnavi (2014) suggesting that *P. aeruginosa* DBTIBNH3 has the ability to fix atmospheric nitrogen into ammonia, which was further confirmed by molecular detection of *nifH* gene. Nitrogenase activity was also quantified for both *S. marcescens* AL2-16 and *S. marcescens* AL6-10 and it was found to be 2.523 and 32.968 nmol ethylene μg^{-1} $protein^{-1}$ hr^{-1} respectively as detected using GC-FID technique. This results was supported by Balachandra *et al*. (2006) where *Serratia* sp. EDA2 isolated from rice could amplify about 750 bp sized partial *nifH* gene, confirming its diazotrophy and its nitrogenase activity was measured by Acetylene Reduction Assay (ARA) technique.

Successful colonization by endophytes depends on many variables, including plant tissue type, plant genotype, the microbial taxon and strain type, and biotic and abiotic environmental conditions (Carroll, 1988). Many bacterial endophytes originate from the rhizosphere environment, which attracts microorganisms due to the presence of root exudates and rhizodeposits. Stem and leaf surfaces also produce exudates that attract microorganisms (Compant *et al*., 2010). Several of these routes involve passive or active mechanisms enabling bacteria to migrate from the rhizoplane to the cortical cell layer, where the plant endodermis represents a barrier for further colonization (Mercado-Blanco, Lugtenberg 2014). For bacteria that can penetrate the endodermis, the xylem vascular system is the main transport route for systemic colonization of internal plant compartments (James *et al*., 2002).

The isolates *S. marcescens* AL2-16, *S. marcescens* AL6-10, *P. aeruginosa* AL2-14B and *B. methylotrophicus* AST5-2 were proved to be real endophytes by performing colonization experiment. These isolates were re-inoculated into the host plant and its population dynamics were observed after 3 DAI and 5 DAI. The colonization of the bacteria in the host tissues also varied with the plant parts. In this study, it was found that the introduced endophyte, *P. aeruginosa* AL2-14B survived in the stem only after 3 DAI with the increased bacterial multiplication by 6×10^2 CFU/g (fresh weight). However, in 5 DAI, the introduced bacteria were found both in stem (21×10^6 CFU/g (fresh weight) and leaves (11.3×10^4 CFU/g (fresh weight). It seems that the bacteria were translocated from stem to leaves through transpiration. This hypothesis is supported by Compant *et al*. (2005) where he reported that the systematic spread of an endophytic *Burkholderia* strain to aerial parts of *Vitis vinifera* seems to be through the transpiration stream. Colonization

of endophytic *P. aeruginosa* AL2-14B on host plant was supported by Gupta *et al*. (2013) where he revealed that the population size of *P. aeruginosa* PM389 gradual increased in root and shoot portion upto 42 day after inoculation (8.6×10^4) and 35 day after inoculation (1.4×10^4) respectively. A significant decreased in the CFU count of *P. aeruginosa* PM389 was observed from 42 DAI in root and from 49 DAI in shoots. There are few reports on experimental greenhouse study of colonization of host plants by bacterial endophytes. For example, *P. aureofaciens* was inoculated and was recovered after 29 days from tall fescue leaves and pea and bean stems, with population of 2.3 log10 CFU/g (fresh weight) (Lamb *et al*. 1996). Fisher *et al*. (1992) recovered bacteria from field-grown sweet corn and reported colonization levels of 2.3 to 6.5 log10 CFU/g (fresh weight) of tissue. Guo and associates (2002) inoculated the roots of hydroponically grown tomato plants with salmonellae at around 4.55 log CFU /ml and, the next day, found that hypocotyls, cotyledons, and stems had around 3 log CFU/ g. Similarly, in this study, the population of *S. marcescens* AL2-16 and *S. marcescens* AL6-10 increased from, 12.4×10^5 to16×10^6 and 56.8×10^5 to 43×10^6, (fresh weight) respectively on stem after 3DAI, but not in leaves. However after 5 DAI, the colonization were reached upto leaves as there were increased in bacterial population. The population of *S. marcescens* AL2-16 on stem and leaves at 5DAI were 93×10^3 and 18.9×10^4. Similarly, the population of *S. marcescens* AL6-10 on stem and leaves at 5DAI were 11×10^5 and 41×10^3. This result was supported by Gyaneshwar *et al*. (2001) where *S. marcescens* IRBG500 showed colonization on rice plant using *gusA* marker, where the colonization was observed after 3 DAI and the bacterial reached to stem and leaves after 6 DAI resulted in significant increased in root length and root dry weight. Rangel de Souza *et al.* (2015) also reported

that *Gluconacetobacter diazotrophicus* efficiently promoted *A. thaliana* plant growth at 50 days after inoculation. The bacterium colonized preferentially root xylem. They found that the bacterial population within *A. thaliana* roots was 1.5×10^6, 3.1×10^6 and 2.1×10^5 CFU/g at 14, 28, and 50 DAI, respectively. At 50 DAI, an increase in number of leaves, leaf area, and shoot and root dry weight was observed. Toumatia *et al*. (2016) established an experiment for colonization of the *Streptomyces mutabilis* strain IA1 and was visualized 10 days post inoculation on the rhizoplane and inside the endorhiza as well as inside tissues of caryopses of plants. Hansen *et al*. (1997) reported that *Pseudomonas fluorescens* strains DF57 and Ag1 colonized barley roots grown in sterile. Here, in this work, results showed that the population of *B. methylotrophicus* AST5-2 increased from, 20.3×10^5 to 10×10^6, (fresh weight) respectively on stem after 3DAI, but not in leaves. However after 5 DAI, the colonization were reached upto leaves as there were increased in bacterial population. The population of *B. methylotrophicus* AST5-2 on stem and leaves at 5 DAI were 7×10^4 and 44×10^3. However, there is no earlier report on colonization of *P. aeruginosa, S. marcescens* and *B. methylotrophicus* in *A. aspera* experimented in green house condition with germ-free plants.

There are few reports, where *Pseudomonas* sp. has been studied as growth enhancement of *Achyranthes*. However, these isolates were isolates of rhizospheric soil of *A. aspera* (Mohinder *et al*. 2011; Misra *et al*. 2012). Here we report an endophytic *P. aeruginosa*, which was isolated from the aerial part of the plant, and promote the growth of the host plant as confirmed by pot trial experiments. The results of pot trials suggest that *P. aeruginosa* AL2-14B is an excellent growth promoter of *A. aspera*, as significant increase in growth parameters of plants was recorded. AL2-14B significantly ($P\leq0.05$)

increased shoot length by 72.83%, fresh shoot weight by 302%, dry shoot weight by 486%, fresh root weight by 385.71%, dry root weight by 700%, area of leaves by 135.28%. Earlier, *Pseudomonas* spp. have been reported as endophytic plant growth promoter in few other plants (Dalal *et al*., 2013; Jasim *et al*., 2013), but not in *A. aspera*. This study also provided the difference between inoculated plant with *S. marcescens* AL2-16 compared with control plant. Used of *S. marcescens* AL2-16 increased the shoot length by 95%, fresh shoot weight by 602%, fresh root weight by 438%, dry root weight by 675% and area of leaves by 127%. Similarly plants inoculated with *S. marcescens* AL6-10 also increased all the growth parameters. This results was supported by Chakraborty *et al*. (2010) where he found that the application of *S. marcescens* TRS-1 promoted growth in tea seedlings as evidence by increased in height, new leaves and leaves biomass. The highest shoot length was observed when plant treated with *S. marcescens* AL2-16 (40.16±0.59) followed by *P. areuginosa* AL2-14B (35.50±0.93) and *S. marcescens* AL6-10 (30.38±1.01). The highest root length was observed when plants treated with *S. marcescens* AL6-10 (30.26±1.35), then *P. aeruginosa* AL2-14B (22.86±1.63) and *S. marcescens* AL2-16 (21.31±0.72). No significant difference was seen between the individual treatment but only had significant differences compared with untreated control plant.

The plants are a major source of antioxidant compounds and several antioxidant compounds have been reported from plants. The significant antioxidant potentials of *A. aspera* L. substantiate its role as an anticancer agent. Endophytic bacteria help in the mediation of reactive oxygen species and anti-oxidant activity in plants. Effect of endophytic bacteria *P. aeruginosa* AL2-14B, *S. marcescens* AL2-16, *S. marcescens* AL6-

10 and *B. methylotrophicus* was studied by conduction several tests including - total phenol content, DPPH scavenging activity, β-Carotene-linoleic acid assay, and reducing power of plant extract. It was observed that inoculation of endophytic isolates, the antioxidant activity of host plant was increased. It has been suggested earlier, that there is a significant impact of endophyte colonization on the anti-oxidant activity in plants, as antioxidant activities are higher for colonized (E+) in comparison to non-colonized (E-) host plants (Hamilton *et al*. 2012). In fact, antioxidants serve to transmit stress signals through the oxidant-antioxidant interaction (Foyer 2005). This may facilitate the chemical communication between a host and asymptomatic endophyte enabling the host to react quickly to pathogenesis and differentiate a pathogen from a mutualist. Changes in host production of antioxidants resulting from endophyte colonization of host tissues have been found in numerous studies. Srinivasan *et al*. (2010) reported high antioxidant activities when *Phyllosticta* sp. cultures were exposed to reactive oxygen species. In the interplay between endophytic bacteria and host plant, the production of both reactive oxygen species and antioxidants may be the mechanism by which the host's hypersensitive and systemic acquired resistance responses are mediated (Tanaka *et al*., 2006). Phenol content was checked in control and treated plants of *A. aspera* L., and the amount of total phenol content in the treated plants by *P. aeruginosa* AL2-14B, *S. marcescens* AL2-16 and *S. marcescens* AL6-10 was found lower than the control plant by only 3.12 %, 12% and 6.24%. However, *B. methylotrophicus* treated plant showed higher phenolic content than control. Kahkonen *et al*. (1999) suggested that there can be no significant correlation between total phenolic content and antioxidant activity of the plant extracts. It is known that different phenolic compounds have different responses in

the Folin-Ciocalteau method. Similarly the molecular antioxidant response of phenolic compounds varies remarkably, depending on their chemical structure. Thus, the antioxidant activity of an extract cannot be predicted only on the basis of its total phenolic content. Hence another three tests were undertaken in this work.

DPPH assay evaluates the ability of antioxidants to scavenge free radicals. Hydrogen-donating ability is an index of the primary antioxidants. These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the phase of lipid oxidation (Lugasi *et al*., 1998). DPPH is a stable free radical with deep purple colour; after receiving proton form a proton donor, such as phenolic compounds, it loses its chromophore and becomes yellow (Sanchez *et al*., 1999). In this study, DPPH activity of the treated plant was found to be higher than the control plant. The free radical scavenging activity of the extract increased with increased in plant extract concentration. The values of DPPH activity of *A. aspera* plants inoculated with *P. aeruginosa* AL2-14B was found to be in a ranged from 9.34 mg/ml to 39.36 mg/ml while the control plants ranged from 5.42 mg/ml to 29.10 mg/ml. *B. methylotrophicus* AST5-2 inoculated plants showed highest values of DPPH among other isolates. It showed the values ranged from 14.27 mg/ml to 76.46 mg/ml. The values of DPPH activity from 11.91 mg/ml to 34.24 mg/ml was given by plants inoculated with *S. marcescens* AL2-16. *S. marcescens* AL6-10 inoculated plants showed similar DPPH values with that of *P. aeruginosa* AL2-14B i.e. from 9.13 mg/ml to 36.52 mg/ml. Kumar *et al*. (2012) reported earlier that the DPPH radical scavenging assay of *A. aspera* extract was 57.53 at 100µg/ml. IC50 is the concentration at which the 50% scavenging activity is obtained. A low IC50 value indicates a high antioxidant activity at a reduced concentration. Here, the

results showed that *B. methylotrophicus* AST5-2 inoculated plant had highest antioxidant activity (IC50-2.743 mg/ml) among other three isolates, followed by *P. aeruginosa* AL2-14B (IC50-6.41 mg/ml), *S. marcescens* AL6-10 (IC50-6.88 mg/ml) and *S. marcescens* AL2-16 (IC50-7.702 mg/ml) while the value of IC50 of control plant was 8.11 mg/ml. Earlier, *A aspera* roots and leaves have been reported to possess DPPH radical scavenging activity with IC50 value of 241.48 µg/ml and 129.91 µg/ml (Jitendra, 2009). Abi Beaulah *et al*. (2011) also reported that DPPH free radical scavenging activity of *A. aspera* leaf was 2.22 at 100 µg/ml of extract. Pandey *et al*. (2014) revealed that *A. aspera* have DPPH free radical scavenging activity with IC50 at a concentration of 62.24 µg/ml. By analyzing the above results, it may be concluded that plants inoculated with selected endophytes had higher antioxidant properties than the untreated control plants. This was in agreement with the finding by Ravindran *et al*. (2012), where he reported that extracts obtained from *Kandelia candel*, *Excoecaria agallocha*, *Rhizophora mucronata* inoculated with *Aspergillus flavus* had the highest DPPH radical scavenging activity with the values of 53±1, 80±0, 72±0 respectively. β-Carotene-linoleic acid assay of extract obtained from inoculated plant was found to be slightly higher than the control plant. The β-carotene bleaching assay is based on loss of the yellow color of β-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion. The rate of beta carotene bleaching is slowed down in the presence of antioxidants. Total antioxidant activity estimated by β-carotene bleaching assay was found to be higher in plant inoculated with *P. aeruginosa* AL2-14B as compared with control plants. The values increased with increased in extract concentration and the values ranged from15.77± 2.52 to 78.85 ± 4.52 with the control having 15.35±2.12 to76.76 ± 4.05.

Reducing power serve as a good indicator of antioxidant potential of the plant. Compounds possessing reducing power are electron donors and can reduce the oxidized intermediates of lipid peroxidation, thus acting as both, primary and secondary antioxidants. In this assay the yellow color of test solution changes to various shades of green and blue, depending upon the reducing power of compound. Presence of reducer converts Fe3+/ferricyanide to ferrous form and its concentration is recorded at 700 nm. The reducing power of *A. aspera* L. leaves inoculated with *P. aeruginosa* AL2-14B was found to be significantly higher than that of control plant. 23% and 18% increase in the reducing power of the extract were recorded at 3.75mg/ml and 5mg/ml of extract concentration respectively. In a different study, Prasad *et al*. (2012) reported that the medicinal plant *Bacopa monniera* treated with root endophytes *Piriformospora indica* DSM 11827 produced higher amounts of bacosides anti-oxidants than untreated control. The endophyte *P. indica* significantly increased the amount of ascorbic acids and elevated the activities of antioxidant enzymes in barley root under stress conditions (Baltruschat *et al*., 2008).