CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. *Achyranthes aspera* L. and its medicinal properties:

Plants have been used as a source of medicine throughout the world for more than 5000 years and still continue to occupy an important place in tradition as well as modern system of medicines. According to the WHO more than 80 % of the world's population relies on traditional herbal medicine for their primary health care. A survey, conducted by Indian Council of Medical Research, revealed that 38% of cancer patients opted for alternative treatments before using allopathic medicines (Chaturvedi *et al*., 2002; Pai 2002). India is a land of rich biodiversity; total number of lower and higher plants in India is about 45,000 species (Uma *et al*., 2010). The traditional knowledge on medicinal plant is the main basis for biocultural and ecosystem conservation as well as selection of various plant species for further pharmacological, photochemical, toxicological and ecological studies (Ramawat *et al*, 2008). Phytochemical examination of a number of plants has been carried out and active ingredients isolated, identified and are currently used as drugs (Ramawat 2008; Balunas 2005; Gurib-Fakim 2006; Chin *et al*., 2006; Canter *et al*., 2005; Merillon *et al*., 2007; Jia *et al*., 2005). In the present era of drug development and discovery of newer drug molecules many plant products are evaluated on the basis of their conventional uses. One of the numerous plants which are being analysed for their therapeutic efficacies is *Achyranthes aspera* L which is commonly known as Latjeera (Hindi) and Rough Chaff tree (English). *Achyranthes aspera* L. is an erect or procumbent, annual or perennial herb, 1-2m in height, often with a woody base (Dey, 2011).

Achyranthes aspera L. (Amaranthaceae) is distributed as weed throughout Tropical Asia, India and other parts of the world. Ayurvedic, Yunani practitioners and Kabirajes use different parts of the plant to treat leprosy, asthma, fistula, piles, arthritis, wound, insect and snake bite, renal and cardiac dropsy, kidney stone, diabetes, dermatological disorders, gynecological disorders, gonorrhea, malaria, pneumonia, fever, cough, pyorrhea, dysentery, rabies, hysteria, toothache etc. The plant is a popular folk remedy in traditional system of medicine throughout the tropical Asian and African countries. The plant is reported to be used as antimicrobial, larvicidal, antifertility, immunostimulant, hypoglycemic, hypolipidemic, anti-inflammatory, antioxidant, diuretic, cardiac stimulant, antihypertensive, anti-anasacra, analgesic, antipyretic, antinoiceptive, prothyrodic, antispasmodic and hepatoprotective (Dey, 2011). Phytochemical screening of ethanolic extract of *Achyranthes aspera* L revealed the presence of triterpenoid saponins possessing oleanolic acid as aglycone, alkaloid achyranthine and steroids. Achyranthine is reported to have anti-inflammatory activity and it has similar mechanism of action as that of steroids i.e. by inhibiting prostaglandin synthesis at phospholipase A2 and at the level of cyclo-oxygenase/PGE isomerase (Uma *et al*., 2010). In Chinese traditional medicine, the hot water extract of the plant has been used as an anti-arthritic and to alleviate arthritic pain (Uma *et al*., 2010). Although it has many medicinal properties, it is particularly used spermicidal (Paul *et al*., 2010), antipyretic (Sutar *et al*., 2008) and as a cardiovascular agent (Neogi 1970). The plant growth promoting properties of endophytes are unique, and therefore it is significant to study such properties from microbial populations linked with medicinally

important and economically important plants. Considering the medicinal importance of *A. aspera*, it's pertinent to understand the role of endophytic bacteria on its growth and other properties.

Pandey *et al*. (2014) conducted an *in vitro* experiment of *A. aspera* L. on antioxidant activity by DPPH free radical scavenging assay, hydroxyl radical scavenging activity, β-Carotene-linoleic acid assay and reducing power assay. It was found out that the total phenol and flavonoid content was 3.363% and 6.36% respectively and IC50 was observed at a concentration of 68.32µg/ml for hydroxyl radical scavenging activity and 62.24µg/ml for DPPH free radical scavenging activity. The results demonstrated that the leaf extract of *Achyranthes aspera* L also possess antibacterial properties which contribute its medicinal values and its potential for cure of skin diseases.

Hassain *et al*. (2013) conducted cytotoxicity test of *A. aspera* leaf extract and showed the highest percentage of mortality (90%) in 1250 µg/ml and LC50 value was 50.12 µg/ml and thrombolytic test showed 32.87 ± 9.42% clot lytic activity for *A. aspera* while positive control (streptokinase) and negative control (water) showed 81.19 ± 3.78% and 6.67 ± 2.58% clot lysis, respectively suggested that *A. aspera* is a good candidate as source of novel anti-tumor agents and thrombolytic drugs.

Rani *et al*. (2012) studied ethanol extract of *A. aspera* L. in its antiobesity potential. In *in vitro* study, the inhibitory activity of EAA on pancreatic amylase and lipase was measured. The antiobesity effect of EAA (900 mg/kg) was assessed in mice fed a high-fat diet with or without EAA for 6 weeks. EAA significantly suppressed the increase in body, retroperitoneal adipose tissue, liver weights, and

serum parameters, namely; total cholesterol, total triglyceride, and LDL-cholesterol level. The anti obesity effects of EAA in high-fat-diet-treated mice may be partly mediated through delaying the intestinal absorption of dietary fat by inhibiting pancreatic amylase and lipase activity.

Umamaheshwari *et al*. (2012) conducted the *in vitro* anticataract and antioxidant activities of aqueous leaves extract of *A. aspera* L. against glucose-induced cataractogenesis using goat lenses. The extracts at a dose of 100 µg/ml and 200 µg/ml were incubated simultaneously with glucose (55 mM) for a period of 72 h and Vitamin E (100 µg/ml) was used as the standard drug. From the results, it was concluded that simultaneous incubation of the plant extracts prevented the preoxidative damage caused by glucose, which is evidenced from the improved antioxidant potential. The aqueous leaves extract of *Achyranthes aspera* protected the lens against glucose-induced oxidative damage which might be helpful in delaying the progression of cataract.

Zambare *et al*. (2011) reported that the 95% ethanol extract of *A. aspera* (EEAA) at a dose of 400 mg/kg showed its effectiveness antidiabetic activity as shown by the reduction in BSL and normalized beta cells. Thus concluded that EEAA served as natural tonic for pancreas in diabetic patients.

Aggarwal *et al*. (2010) studied the alternative treatment of kidney stone by investigated *A. aspera*. and its inhibitory potential on nucleation and the growth of the calcium oxalate (CaOx) crystals as well as on oxalate-induced cell injury of NRK 52E renal epithelial cells *in vitro* was investigated. *A. aspera* extract exhibited a concentration-dependent inhibition of the growth of CaOx crystals. *A. aspera* extract

prevented the injury of NRK 52E cells in a dose-dependent manner. The cell viability increased with the different concentrations of the plant. The lactate dehydrogenase (LDH) release decreased in a concentration-dependent manner. These studies indicated that *A. aspera* extract has a potential to inhibit both nucleation and the growth of the CaOx crystals and can prove to be a potent candidate for phytotherapy against urolithiasis.

Elumalai *et al*. (2009) reported that the ethanol extract of the leaves of *A. aspera* L. elevated antifungal activity against *C.kefyr, Cryptococcus neoformans, Aspergillus niger* and *A. flavus*. The methanol extract showed antifungal activity against *Cryptococcus neoformans* and *A. flavus*. Thus it revealed a significant scope to develop a novel broad spectrum of antifungal herbal formulation.

Alam *et al*. (2008) conducted an experiment based on the analgesic and central nervous system depressant activity of the methanol extract of *A. aspera* L. and found out that 70% metanol extract of *A. aspera* produced rapid onset and maximized the duration of sleeping time when administered with thiopental sodium. The extract decreased the motor activity and exploratory behavior of mice suggested its effectiveness in analgesic and central nervous system depressant activity. It justified its use in folkloric remedies.

Shibeshi *et al*. (2006) conducted anti-fertility activities and safety evaluations of crude extracts of *A. aspera* and found out the extract prolonged estrous cycle, estrous and metestrous phases ($p < 0.05$) of rats. There was reduction in weight of ovary however uterus weight was increased ($p < 0.05$). The study hinted that the methanolic extract has anti-fertility effect and is safe at the contraceptive doses.

2.2. Plant growth promoting endophytic bacteria and their biodiversity:

Bacterial endophytes colonize the internal tissues of plants which are found in nearly every plant worldwide. Most of the endophytes are able to promote the growth of plants. The mechanisms of plant growth-promotion known to be utilized by bacterial endophytes are similar to the mechanisms used by rhizospheric bacteria, i.e. uptake the nutrients needed for plant growth and modulation of plant growth and development. Endophytic bacteria act to facilitate plant growth in agriculture, horticulture and phytoremediation. Comparison of genome between bacterial endophytes and rhizospheric plant growth-promoting bacteria are starting to reveal potential genetic factors involved in an endophytic lifestyle, which should facilitate a better understanding of the functioning of bacterial endophytes (Santoyo *et al*., 2016). Hardoim *et al*., (2015) described endophytes as "all microorganisms that spend at least parts of their life cycle inside plants". Endophytic bacteria have been isolated from different plant tissues such as roots (Xia *et al*., 2016; Abbamondi *et al.*, 2016), stem (Andreolli *et al*., 2016; Ji *et al*., 2014), barks (Khan *et al*., 2016; Doley and Jha, 2014), shoots (Taghavi *et al*., 2009), leaves (Ji *et al*., 2014), fruits (Abdallah *et al*., 2016), rhizomes (Kumar *et al*., 2016) and seeds (Herrere *et al*., 2016).

The beneficial effects of endophytic bacteria have been attributed to their ability to produced bioactive compounds and enzymes to survive in the unique chemical environment of the host plant (Ali *et al*., 2016). Their metabolic activities also help in increasing the growth and development of plants. This is because of direct growth promotion effects through the production of plant growth regulators, synthesis of ACC (1 aminocyclopropane-1-carboxylate) deaminase (Taghavi *et al*., 2009; Khan *et*

al., 2016), phosphate solubilization, siderophore production (Natul *et al*., 2013; Abbamondi *et al*., 2016), N_2- fixation (Szilagyi-Zecchin *et al*., 2014), and/ or by indirect mechanism by providing resistance to diseases through the production of antimicrobial metabolites to inhibit pathogenic microorganisms (Kumar *et al*., 2016). Ali *et al*. (2014) reported that a group of genes are responsible for the endophytic lifestyle. The endophytic bacteria have genome difference compared to rhizospheric bacteria. Endophytic bacteria have been isolated from a large diversity of plants was reviewed by Santoyo *et al*. (2016). He suggested that 300,000 species of plants found on the earth contains one or more endophytes.

Microorganisms which are mainly considered as plant growth promoting endophytes include the strains in the bacterial genera of *Acinetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azospirillium, Azotobacter, Azomonas, Bacillus, Beijerinckia, Burkholderia, Clavibacter, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Methylobacterium, Microbacterium, Pantoea, Plantibacter, Pseudomonas, Rhodococcus, Rhizobium, Serratia, Sphingomonas* and *Stenotrophomonas*.(Abbamondi *et al*., 2016; Andreolli *et al*., 2016; Khan *et al*., 2016; Kumar *et al*., 2016; Li *et al*., 2016; Lumactud *et al*., 2016).

Andreolli *et al*. (2016) investigated ecology of endophytic bacteria isolated from 3 and 15 year-old vine stems of *Vitis vinifera cv. Corvina*. The analysis was performed by means of culture-dependent techniques. The obtained results showed *Bacilli* and *Actinobacteria* are frequently isolated from 3 year-old plants, whereas Alpha- and Gamma- Proteobacteria classes are more prevalent in the 15 year-old plants.

Herrera *et al*. (2016) surveyed the presence of bacterial endophytes in the seeds of wheat grown in Argentina. They were screened for plant growth promotion activity and biocontrol abilities against *F. graminearum.* Four isolates were assignated to *Paenibacillus* genus, one isolate to *Enterobactereaceae* of *Pantoea* genus. Khan *et al*. (2016) revealed the 5 endophytic bacteria, in which 2 strains were from *Sphingomonas* sp.; 2 strains from *Bacillus* sp. and 1 from *Methylobacterium* genus isolated from the medicinal plant, *Moringa peregrine*. Recent molecular studies on endophytic bacterial diversity have revealed a large richness of species. Endophytes promote plant growth and yield, suppress pathogens, may help to remove contaminants, solubilize phosphate, or contribute assimilable nitrogen to plants. Some endophytes are seedborne, but others have mechanisms to colonize the plants that are being studied. Bacterial mutants unable to produce secreted proteins are impaired in the colonization process. Plant genes expressed in the presence of endophytes provide clues as to the effects of endophytes in plants. Molecular analysis showed that plant defense responses limit bacterial populations inside plants (Rosenblueth and Martínez-Romero, 2006).

Jasim *et al*. (2013) reported the presence of four different endophytic bacterial strains from ginger rhizome in which the isolate ZoB2, identified as *Pseudomonas* sp. was found to have the ability to produce IAA, ACC deaminase and siderophore. By considering these plant growth promoting properties, ZoB2 was expected to have considerable effect on the growth of ginger.

2.3. Indole Acetic Acid (IAA) production by endophytic bacteria:

Plant growth regulators play key roles in plant growth and development and in the response of plants to their environments. During its lifetime, a number of nonlethal stresses acquired by a plant that limit its growth until either the stress is removed or the plant is able to adjust its metabolism to overcome the effects of the stress. When plants encounter growth limiting environmental conditions, they try to adjust the levels of their endogenous plant regulators in order to decrease the negative effects of the environmental stressors. While this strategy is sometimes successful, PGPB may also produce plant growth regulators under *in vitro* conditions altering the plant regulator's levels, thereby affecting the plant's hormonal balance and its response to stress (Glick, 2012).

Auxin, primarily in the form of indole-3-acetic acid (IAA), has been known to be a regulator of plant growth and development (Sibbon *et al*., 2000). It is involved in processes as diverse as branching, stimulates seed and tuber germination, xylem and root development, controls the processes of vegetative growth, initiates growth of lateral and adventitious roots, mediates plant responses to light and gravity, florescence and fructification of plants and also affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions (Frankenberger and Arshad, 1995; Davies, 2004; Tsakelova *et al*., 2006). IAA also stimulates cell division, cell enlargement and root initiation (Salisbury, 1994). IAA produced by PGPB increases the plant rooting and enhances plant mineral uptake. The root exudation helps in stimulating bacterial colonization which amplifies the inoculation effect of the plants (Lambrecht *et al*., 2000; Spaepen *et al*., 2007).

L-Tryptophan (Tryp) has been considered as the main IAA precursor of IAA biosynthesis pathway (Normanly *et al*., 1995; Bartel 1997). It is accepted that microbes associated with plant possess different routes for IAA biosynthesis. There are four major tryptophan (Tryp)-dependent pathways and one Tryp-independent pathway (Lin and Xu, 2013). IAA biosynthesis in plants may occur via different pathways, according to their intermediates: indole-3-acetamide (IAM), indole-3-pyruvate (IPyA), tryptamine, and indole-3-acetonitrile. The two most common routes for IAA biosynthesis in bacteria are the IAM and the IPyA pathways.

Most beneficial bacteria synthesize IAA via the IPyA pathway (Lambrecht *et al*., 2000). Production of IAA by the IPyA pathway has been reported in PGPB strains such as *Enterobacter cloacae, Erwinia herbicola, Bradyrhizobium elkanii* and *Azospirillum brasilense* (Zimmer *et al*., 1998; Koga *et al*., 1991; Minamisawa *et al*., 1996; Brandl and Lindow, 1996). The indole-3-acetamide (IAM) pathway is the well known pathway in bacteria. This pathway has been reported for various bacteria such as *Pantoea agglomerans, Bradyrhizobium* sp., *Rhizobium* sp. and *Pseudomonas chlororaphi* (Sekine *et al*., 1989; Morris, 1995; Dimkpa *et al*., 2012; Theunis *et al*., 2004). *Streptomyces albidoflavus* and *Streptomyces* sp. En-1 (Legault *et al*., 2011; Manulis *et al*., 1994; Narayana *et al*., 2009; Xu and Lin, 2013).

Chimwamurombe *et al*. (2016) isolated 73 putative endophytic bacteria from marama bean and were identified as *Rhizobium, Massilia, Kosakonia, Pseudorhodoferax, Caulobacter, Pantoea, Sphingomonas, Burkholderia, Methylobacterium, Bacillus,* Actinobacteria, *Curtobacterium, Microbacterium Mucilaginibacter, Chitinophaga*. Screening for plant growth-promoting activities

revealed that the isolates showed production of IAA, This was the first report that marama bean seeds may harbor endophytes that can be cultivated from seedlings which have potentially plant growth promoting activities.

Khan *et al*. (2016) reported that the endophytic bacteria *Bacillus subtilis* LK14 isolated from bark of *Moringa peregrine* showed significantly higher IAA production by spectrophotometry analysis method. The analysis showed that LK14 produced the highest (8.7 μM) IAA on 14th day of growth. It was applied to *Solanum lycopersicum*, where it significantly increased the shoot and root biomass and chlorophyll (a and b) contents as compared to control plants.

Li *et al*. (2016) collected 16 endophytic bacteria strains from the roots of elephant grass. Out of these, four strains, pp01, pp02, pp04, and pp06 were selected and tested *in vitro* for their plant growth promoting properties and its effects on plant growth and salt stress tolerance of *Hybrid pennisetum*. The results showed that each strain tested had the ability to produce IAA at a range of 10.50–759.19 mg/l, where pp06 showed the highest value (759.19 mg/l) followed by pp04 (40.88 mg/l), pp01 (19.85 mg/l) and pp02 (10.50 mg/l).

Yu *et al*. (2016) studied the population density of endophytic bacteria varied irrespective of crops, sampling times and soil amendments. A total of 119 and 277 bacterial isolates were isolated from soybean and corn roots, respectively. 39.6% of the total isolates showed IAA production in the range of 1-23 μg m/l in culture medium supplemented with tryptophan. Fourteen isolates, named as S1-S4 from soybean roots and C1-C10 from corn roots, had IAA activity over 10 mg/l.

Kumar *et al*. (2016) suggested that fourteen endophytic bacterial isolates were isolated from the rhizome of *Curcuma longa* L. and were identified to six strains namely *Bacillus cereus* (ECL1), *Bacillus thuringiensis* (ECL2), *Bacillus* sp. (ECL3), *Bacillus pumilis* (ECL4), *Pseudomonas putida* (ECL5), and *Clavibacter michiganensis* (ECL6). All the endophytic bacterial strains produced IAA with maximum (23 µg/ml) in *P. putida* (ECL5) and minimum (14 µg/ml) in *Clavibacter michiganensis* (ECL6) on supplementation of 400 L-tryptophan (µg/ml) and remaining four strains value of IAA were in between them.

Abbamondi *et al*. (2016) reported that endophytes from tomato plant root, genotypically characterized as *Pseudomonas* sp., *Rhodococcus* sp., *Agrobacterium* sp. were able to produced IAA which increases in the formation of root hairs of the inoculated plants. This effect was seen for all *A. thaliana* seedlings inoculated with the isolated endophytes. Prasad *et al*. (2014) isolated 6 endophytic bacteria from avocado (G1N, G2N, G1O) and black grapes (SA3, SA4, A1) which gave positive result for IAA production where maximum production was exhibited by strain SA3 (54.83 µg/ml).

Rangjaroen *et al*. (2014) investigated that out of 250 bacterial strains obtained from different tissues of rice plant, 21 strains were positive for IAA production. Among these *Sphingomonas* sp. PS5 gave maximum production of IAA (16.16±1.59 µg/ml).

Patel and Patel (2014) isolated *Pseudomonas stutzeri* from plant root grown in saline desert which has the ability to produced IAA (97.5 µg/ml) with the supplement of 200 µg/ml of L-tryptophan, incubated for 168 h. Jasim *et al*. (2014) studied the

presence of four different endophytic strains isolated from the rhizome of ginger and was identified as *Bacillus* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., and *Staphylococcus* sp. Among the four endophytic isolates, ZoB2 (*Pseudomonas* sp.) was found to have the ability to produce IAA.

Uma Maheshwari *et al*. (2013) isolated endophytic bacteria from various tropical grain legume crops namely redgram, blackgram, greengram, cowpea and chickpea and were identified as *Bacillus* sp., *Micrococcus* sp., *Pseudomonas* sp., *Flavobacterium* sp. and *Serratia* sp. All the isolates were evaluated for the production of phytohormones viz. gibberellic acid (GA), indole acetic acid (IAA) and cytokinin. The endophytic isolates produced GA from 0.75 to 2.83 g/ml, had an IAA activity between 0.12 g/ml to 6.46 g/ml and cytokinin values were 0.52 g/ml to 2.96 g/ml. Hence this study clearly establishes the beneficial effect of bacterial endophytes on tropical legumes. Liu *et al*. (2010) reported that *Serratia* sp. G3 isolated from the stem of wheat has the ability to produced the plant growth hormone indole-3-acetic acid.

Ambawade and Pathade (2013) isolated a novel endophytic bacterium from the root of the banana (musa spp), which was identified as *Stenotrophomonas maltophilia* and named as BE-25. All the twenty two isolates were screened for their productivity of indole acetic acid (IAA) by salkowski's method and BE-25 isolate showed highest amount of indole acetic acid production supplemented with and without L-tryptophan in medium. IAA production of BE-25 was further confirmed by thin-layer chromatography as well as by high-performance liquid chromatography.

Sgroy *et al.* (2009) designed to isolate and characterize endophytic bacteria from roots of halophyte *Prosopis strombulifera* which grown under extreme salinity and to evaluate *in vitro* test related to plant growth promotion or stress homeostasis regulation. A total of 29 endophytic strains which were grouped into seven according to similarity were able to grow and produce phytohormone. IAA production was significantly higher for *Bacillus subtilis* (Ps8), *Bacillus pumilus* (Ps19), and *Pseudomonas putida* (Ps30), which produced 0.5; 0.7, and 2.2 µg/ml, respectively, compared with Ps7 (*Lysinibacillus fusiformis*), Ps14 (*Bacillus licheniformis*), and Ps27 (*Achromobacter xylosoxidans*), which produced less than 0.1 µg/ml, and Ps9 (*Brevibacterium halotolerans*) failed to produce.

Janarthine and Eganathan (2012) isolated endophytic *Sporosarcina aquimarina* SjAM16103 from the inner tissues of pneumatophores of mangrove plant *Avicennia marina* which produced 2.37 μMol/ml of indole acetic acid. This isolate could solubilize phosphate molecules and fixed atmospheric nitrogen. The growth promoting activity and its role in host plants were analysed for endophytic *S. aquimarina* SjAM16103 and found out that the growth of endophytic *S.aquimarina* SjAM16103 inoculated explants were highly significant than the uninoculated control explants. Root hairs and early root development were observed in the endophytic *Sporosarcina aquimarina* SjAM16103 inoculated explants.

2.4. Siderophore production by endophytic bacteria:

Iron is a vital nutrient and occurs as Fe^{3+} in the aerobic environment and forms insoluble hydroxides and oxyhydroxides. These insoluble forms are not accessible to both plants and microbes. Iron (Fe3+) is biologically important being a constituent of

cytochrome and others heme or non heme proteins and also a co-factor in various enzymes (Macagnan *et al*., 2008). Both plants and microbes have significantly high Fe requirements of 10^{-4} to 10^{-9} M and 10^{-5} to 10^{-7} M respectively and this condition is more in the rhizosphere where plants, bacteria, and fungi compete for iron (Guerinot and Ying, 1994; Loper and Buyer, 1991).

Microorganisms growing under aerobic conditions need Fe for a variety of functions including reduction of ribotide precursors of DNA, reduction of oxygen for synthesis of ATP, formation of heme, and, for other essential purposes. At least one micromolar (µM) Fe is required for optimum growth. These environmental restrictions and biological imperatives makes microorganisms to form specific molecules which may compete effectively with hydroxyl ion for the Fe^{3+} state of Fe, a nutrient which is rich but essentially unavailable (Neilands, 1995).

Endophytes synthesize low molecular weight compounds termed as siderophores (400–1500 Da) that sequester Fe^{3+} since they have high Fe^{3+} affinity constants (K_a, $10^{23}$to 10^{52}) and mobilize the irons present (Zhang *et al*., 2008 ; Vendan *et al*., 2010). The siderophores are water soluble and of two types, viz., extracellular and intracellular, i.e., secreted as iron-free siderophores for cellular iron uptake and located within the cell for intracellular iron storage, respectively (Johnson *et al.*, 2013). Based on their functional groups, structural features and types of ligands, bacterial siderophores have been classified into three main classes: catecholates hydroxamates and carboxylates (Crowley, 2006). There are more than 500 known siderophores, of which 270 of them were determined their chemical structures (Hider and Kong, 2010). Chhibber *et al*. (2008) reported the production of ornibactin type

siderophore by *Stenotrophomonas maltophilia* and Ryan *et al*. (2009) mentioned its ability to produce the catechol type siderophore compound enterobactin based on their recently sequenced genomes.

Siderophores producing bacteria sequestrate the limited iron and thereby reduce its availability for growth of phytopathogens. Thus, they enable the plant growth promotion indirectly (Alexander and Zeeberi, 1991). Kumar *et al*. (2016) isolated 14 endophytic bacteria from the rhizome of *Curcuma longa* L. Out of these only two strains namely *Bacillus* sp. ECL3 and *Pseudomonas putida* ECL5 produced siderophore which was confirmed by the development of yellow orange halo zone around the bacterial spot.

Abbamondi *et al*. (2016) isolated 12 endophytes from the roots of tomato and were characterized as *Pseudomonas* sp, *Rhizobium* sp, *Rhodococcus* sp, and *Agrobacterium* sp. The isolates were allowed to grow in liquid 284 medium with different concentration of iron concentration (0 µM, 0.25 µM and 3 µM). Most of the isolates showed highest siderophore activity both at 0 µM and 0.25 µM of iron concentration but found reduced at 3 µM of iron concentration. Prasad *et al*. (2014) reported that 3 isolates from avocado (G2N, SA3, SA4) and and 1 (A1) from black grape gave positive result for siderophore production.

Jasim *et al*. (2014) demonstrated the diverse community of endophytic bacteria associated with ginger rhizome. Among the four endophytic isolates, ZoB1 (*Bacillus sp*.), ZoB2 (*Pseudomonas* sp.) and ZoB3 (*Stenotrophomonas* sp.) were found to have the ability to produce siderophore. Different species of *Bacillus* have been reported to produce of siderophores (Gardner *et al*., 2004; Wilson *et al.*, 2010).

Gupta *et al*. (2013) isolated endophytic *P. aeruginosa* PM389 from the healthy pearl millet plant which produced catecholate type of siderophore (63.5% units) which exhibited siderophore index of 0.711. 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.

Khilyas *et al*. (2016) reported that two strains of *S. marcescens* SM6 and SR41-8000 which 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* can also produced siderophore. Seyedsayamdost *et al*., 2012 reported that a new *Serratia* sp. designated as *Serratia* sp. v4 that produced serratiochelin and an analog of serratiochelin.

Rungin *et al*. (2012) experimented siderophore-deficient mutant of *Streptomyces* sp. GMKU 3100 by inactivation of the gene *desD*, which codes for a siderophore synthetase. The pot trial experiments was conducted to evaluate the influence of the siderophore-producing endophytic *Streptomyces* sp. GMKU 3100 on plant growth promotion in rice plants in comparison with the siderophore-deficient mutant. There was significant increase of root and shoot biomass as well as root and shoot lengths when wild type strain was applied when compared with the untreated control and the siderophore-deficient mutant. The results concluded that the enhancement of plant growth was due to siderophore production from *Streptomyces* sp. GMKU 3100. Enhancement of plant growth by endophytic *Streptomyces* is also supported from

other experiments e.g. tomato (Tan *et al.,* 2006), cowpea (Dimkpa *et al*., 2008), neem tree (Verma *et al*., 2011) and chickpea (Misk and Franco, 2011).

Patel *et al*. (2012) isolated 18 endophytic bacteria from the stem and roots of *Lycopersicon esculentum* in which five isolates namely HR1, HR3, HR4, HR7 and HR8 showed production of siderophore. Janarthine and Eganathan (2012) isolated endophytic *S. aquimarina* SjAM16103 from the inner tissues of *pneumatophores* of mangrove plant *Avicennia marina* which produced siderophore, confirmed by forming halo zone on CAS agar.

Sgroy *et al*. (2009) reported that a total of 29 endophytic strains were isolated from the roots of *Prosopis strombulifera*. Out of these, only one isolate *Pseudomonas putida* Ps30 able to produce siderophore.

Macagnan *et al*. (2008) reported that the production of siderophores appears to be involved in inhibiting basidiospore germination by the culture supernatants of *Streptomyces albovinaceus, Streptomyces griseus,* and *Streptomyces virginiae,* because the supernatants from Fe^{3+} deficient medium showed lower levels of germination compared those from Fe^{3+} supplemented medium. The siderophores of these species appear to have high Fe^{3+} complexing capacity, depriving the pathogen of this nutrient.

2.5. Phosphate solubilization by endophytic bacteria:

Phosphorus is the most important key element in the nutrition of plants, next to nitrogen. Its plays an important role in energy transfer signal transduction, photosynthesis processes, cell division, macromolecular biosynthesis, biological

oxidation, growth, respiration, reproduction and nutrient uptake in plants (Khan *et al*., 2010; Sashidhar and Podile, 2010). Although phosphorus is abundant in soils in both inorganic and organic forms, it is a major limiting factor for plant growth as it is an unavailable form for root uptake. Inorganic phosphate occurs in soil, mostly in insoluble mineral complexes, some of them appearing after frequent application of chemical fertilizers. These insoluble, precipitated forms cannot be absorbed by plants (Rengel and Marschner, 2005). Organic matter is also an important reservoir of immobilized phosphorus that accounts for 20-80% of phosphorus in soils (Richardson 1994). Despite the abundance of phosphorus in the soil (often as high as 400 to 1,200 mg/ kg of soil), only 0.1% of the total phosphates exists in a soluble form available for uptake by plants (Zhou *et al*., 1992). Moreover the efficiency of applied P fertilizers in chemical form rarely exceeds 30% due to its fixation, either in the form of iron/aluminium phosphate in acidic soils (Norrish and Rosser, 1983) or in the form of calcium phosphate in neutral to alkaline soils (Lindsay *et al*., 1989). In addition, phosphate (P) fertilizers applied in agricultural fields are quickly washed away by rain thereby polluting ground water and rivers (Hamdali *et al.*, 2008) and only 10–30% of applied phosphate fertilizer is taken up by plants (Mclaughlin *et al*., 1988).

Microorganisms are an integral component of the soil phosphorus cycle and are important for the transfer of phosphorus between different pools of soil. Phosphate Solubilizing Bacteria (PSB) through various mechanisms of solubilization and mineralization are able to convert inorganic and organic phosphate into the bioavailable form facilitating uptake by plant roots (Khan *et al*., 2009). Thus, PSB is an important trait in PGPB as well as in plant growth-promoting activity (Richardson,

2001; Rodríguez and Fraga, 1999). The use of PGPB with P-solubilizing abilities in agricultural soils is considered as an environment-friendly alternative to synthetic phosphorus fertilizers.

The ability of PGPB to solubilize mineral P has been of great interest to agriculture as it lead to enhanced availability of phosphorus for effective plant growth. PGPB have been reported to activate precipitated phosphate to plants, thereby representing a possible mechanism of plant growth promotion under field conditions (Bhattacharyya and Jha, 2012). Bacteria that have been reported as P-solubilizers include *Pseudomonas, Bacillus, Rhodococcus, Arthrobacter, Serratia, Chryseobacterium, Gordonia, Phyllobacterium, Delftia* (Wani *et al*., 2005; Chen *et al.,* 2006), *Azotobacter* (Kumar *et al.*, 2001), *Xanthomonas* (De Freitas *et al*., 1997), *Enterobacter, Pantoea* and *Klebsiella* (Chung *et al*., 2005).

The main phosphate solubilization mechanisms employed by microorganisms include: (1) production of complexing or mineral dissolving compounds e.g. organic acid anions, siderophores, protons, hydroxyl ions, CO_2, (2) production of extracellular enzymes and (3) the liberation of phosphate during substrate degradation (McGill and Cole, 1981). Inorganic phosphate solubilization by P-solubilizing microorganisms occurs mainly by organic acid release either by lowering the pH or by enhancing chelation of the cations bound to phosphate. The lowering in pH of the medium suggests the release of organic acids by the P-solubilizing microorganisms (Whitelaw 2000; Maliha *et al*., 2004) via the direct oxidation pathway that occurs on the outer face of the cytoplasmic membrane (Zaidi *et al*., 2009). These acids are formed by the metabolism of microbial activity, specially by oxidative respiration and fermentation

of organic carbon sources (e.g., glucose) (Atlas and Bartha, 1997; Trolove *et al*., 2003). The prominent acids released by PSB in the solubilization of insoluble phosphate are gluconic acid (Di-Simine *et al*., 1998; Bar-Yosef *et al*., 1999), oxalic acid, citric acid (Kim *et al*., 1997), lactic acid, tartaric acid, aspartic acid (Venkateswarlu *et al*., 1984).

Plant growth promoting endophytic bacteria have been reported to solubilize inorganic phosphate and promote the growth of turmeric plants. For example, Kumar *et al*. (2016) reported that *Bacillus cereus* ECL1, *Bacillus* sp. ECL3, *Bacillus pumilis* ECL4, *Pseudomonas putida* could solubilize inorganic phosphate. These strains stimulated shoot and root growth in turmeric planlets under gnotobiotic conditions. Growth promotion was correlated with significant increases in nitrogen (N) and Phosphorus contents of the plant tissues. Similarly, *Klebsiella pneumonia* KW7-S06, KW7-S22, KW7-S33 isolated from the rice also solubilized inorganic phosphate and significantly promoted the growth of rice plants. (Ji *et al*., 2014).

Audipudi *et al*. (2014) isolated endophytic *Curtobacterium* CEG, a novel strain from green fruit of chilli and found out that the level of phosphate solubilization was 356.65µg/ml. Similarly, 19 endophytic bacteria were isolated from the red fruit of chilli and among these, 10 isolates namely, CEFR-1, CEFR-3, CEFR-4, CEFR-5, CEFR-6, CEFR-7, CEFR-9, CEFR-10, CEFR-11 and CEFR-12 were found to be positive in phosphate solubilization (Allu *et al*., 2014).

Lins *et al*. (2014) isolated 31 endophytic bacteria from cashew leaves. Out of these, only 3 strains namely BC-51 (36.73±1.00µg/ml), BC-52 (113.53±15.99µg/ml) and BC-53 (1569.41±11.00µg/ml) could solubilized phosphate.

Jasim *et al*. (2013) resulted in the identification of endophytic bacteria namely, PnB 1 (*Bacillus* sp.1), PnB 8 (*Klebsiella pneumoniae*) and PnB 9 (*Enterobacter* sp.) isolated from *Piper nigrum* with the ability to utilize phosphate as confirmed by forming halozone on pikovskaya agar medium. Studies of Datta *et al*. (1982) confirms the role of *Bacillus firmus* in the phosphate uptake of wheat and rice plants. Chaiharn and Lumyong (2009) suggested that organisms belonging to *Klebsiella* sp. are also able to utilize phosphate. The report of Lopez *et al*. (2011) shows the ability of endophytic *Enterobacter* sp. to solubilize phosphate on a large.

Thamizh Vendan *et al.* (2010) reported that 9 out of 18 endophytic isolates from gingseng plants had phosphate solubilizing ability by detecting extracellular solubilization of precipitated tricalcium phosphate with glucose as sole source of carbon. Long *et al.* (2008) isolated seventy-seven endophytic bacterial isolates from roots, stems and leaves of black nightshade plants (*Solanum nigrum*) grown in two different native habitats in Jena, Germany and six isolates were able to solubilize inorganic phosphate.

2.6. Nitrogen fixation by endophytes:

Nitrogen is an important limiting factor for the growth of plant in various environmental conditions. Despite abundance of atmospheric nitrogen (78%), it cannot be utilized for growth and metabolism. It must be reduced to ammonia for use by any organisms by a process called nitrogen fixation. Application of industrially manufactured nitrogen fertilizer has been one of the most popular ways to provide nitrogen nutrition to the plants to attain high crop productivity. However, excessive and continuous use of chemically synthesized fertilizer may lead to several

consequences which include: (i) grown water contamination of nitrate due to leaching and dentrification. (ii) surface water contamination by eutrophication. (iii) production of greenhouse gases during manufacture of nitrogen fertilizer (Bhattarcharjee *et al*., 2008).

Any bacterium could be considered as an endophytic diazotroph if (i) it can be isolated from disinfected plant tissue (ii) it proves to be situated inside the plant by *in-situ* identification and (iii) it fix nitrogen. This definition includes internal colonists with apparently neutral or saprophytic behavior as well as symbionts (Hartmann *et al.,* 2000). Endophytic bacteria are better than their rhizospheric and rhizoplanic counterparts in terms of benefiting their host through nitrogen fixation as they can provide fixed nitrogen directly to their host (Cocking 2003). As low partial oxygen pressure is necessary for the expression of the O_2 sensitive enzyme, nitrogenase, endosphere of plant root is more amenable for N_2 - fixation reaction. 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).

Endophytic N_2-fixing bacteria appear to represent only a small proportion of total endophytic bacteria (Barraquio *et al*., 1997; Ladha *et al*., 1983; Martínez *et al*., 2003) and increasing N_2-fixing populations in plants has been measured as a possibility to increase nitrogen fixation. Nitrogen-fixing bacteria were identified in sweet potato in N-poor soils with an analysis that consisted of amplifying nitrogenase (*nifH*) genes by polymerase chain reaction (Reiter *et al*., 2003). The resulting sequences, presumably derived from endophytes, resembled those from rhizobia, including *Sinorhizobium*

meliloti, *Sinorhizobium* sp. strain NGR234, and *Rhizobium etli*. Other detected bacteria were *Klebsiella* sp. and *Paenibacillus odorifer* (Reiter *et al*., 2003).

Kallar grass grows in N-poor soils in Pakistan have a diversity of *Azoarcus* sp. which have been recovered from it (Reinhold-Hurek *et al*., 1993). *Klebsiella* sp. strain Kp342 also found from wheat plant which fixes N_2 (Iniguez *et al*., 2004) that increases maize yield in the field (Riggs *et al*., 2001). Similarly, nitrogen fixing endophytes seem to reduce N deficiencies of sweet potato (*Ipomoea batatas*) in N-poor soils (Reiter *et al*., 2003). It is evident from the reports that the *Gluconoacetobacter diazotrophicus* (*Acetobacter diazotrophicus*) is the main contributor of endophytic biological nitrogen fixation in sugarcane, and it has the ability to fix the N approximately 150 Kg N ha $^{-1}$ year $^{-1}$ (Dobereiner *et al*., 1993; Muthukumarasamy *et al*., 2005).

Ji *et al*. (2014) isolated 576 endophytic bacteria from the leaves, stems, and roots of 10 rice cultivars and identified 12 of them as diazotrophic bacteria using a specific primer set of *nif* gene. Through 16S rDNA sequence analysis, *nifH* genes were confirmed in the two species of *Penibacillus*, three species of *Microbacterium*, three *Bacillus* species, and four species of *Klebsiella.* Rice seeds treated with these plant growth-promoting bacteria (PGPB) showed improved plant growth, increased height and dry weight and antagonistic effects against fungal pathogens.

Szilagyi-Zecchin *et al*. (2014) reported that six endophytic bacteria of corn roots, identified as *Bacillus* sp. and as *Enterobacter* sp. by sequencing of the 16S rRNA gene showed an indication of the nitrogen fixation by the formation of the typical pellicule in semi-solid N-free medium. However, in only four strains (CNPSo 2476,

CNPSo 2477, CNPSo 2478 and CNPSo 2480) the *nifH* gene was amplified. *Bacillus* CNPSo 2477 and CNPSo 2476 were positive for *nifH* gene amplification (primers *nifHF* and *nifHI*), producing an amplified fragment of about 780 bp. *Enterobacter sp.* CNPSo 2480 was positive for the two pairs of primers tested (*nifHF* and *nifHI*; *nifH-F* and *nifH-R*). *Bacillus* sp. CNPSo 2478, amplified with the second primer, resulted in a fragment of about 400 bp. These strains were also positive to the ARA test. The nitrogen fixation genes are found in different phylogenetic groups (Affourtit *et al*., 2001).

Balachandar *et al*. (2006) isolated several endophytic diazotrophs from rice and screened for their nitrogenase assay by ARA test and amplification of partial *nifH* gene. The experiment was assayed to see the ability of diazotrophic endophytes, *Serratia* sp. whether to colonize the rice seedlings grown in the presence of flavonoids and growth hormones, under gnotobiotic condition using a strain marked with transposon based egfp and Km^r. The colonization was checked through re-isolation from different parts of rice seedlings in LB+Km plates. It was found out that population and in planta nitrogenase activity of *Serratia* in rice seedlings were significantly increased and the inoculation of *Serratia* sp. with flavonoids increased the plant biomass and biochemical constituents of rice seedlings under controlled condition.

Sgroy *et al*. (2009) reported that a total of 29 endophytic strains were isolated from the roots of *Prosopis strombulifera*. Out of these, seven selected strains were evaluated nitrogen fixation *in vitro* based on the growth on nitrogen-free medium, all the seven isolates namely Ps7 (*Lysinibacillus fusiformis*), Ps8 (*Bacillus subtilis*), Ps9

(*Brevibacterium halotolerans*), Ps14 (*Bacillus licheniformis*), Ps19 (*Bacillus pumilus*) Ps27 (*Achromobacter xylosoxidans*), Ps30 (*Pseudomonas putida*) showed capacity to grow in nitrogen-free medium.

Han *et al*. (2005) isolated a novel, plant growth-promoting bacterium *Delftia tsuruhatensis*, strain HR4 from the rhizoplane of rice (*Oryza sativa* L., cv. Yueguang) in North China and showed a high nitrogen-fixing activity in N-free Dobereiner culture medium. The acetylene reduction activity and $^{15}N_2$-fixing activity (N_2FA) were 13.06 C_2H_4 nmol ml^{-1} h^{-1} and 2.052 15Na.e.%, respectively. The *nif* gene was located in the chromosome of this strain.

Kuklinsky-Sobral *et al.* (2004) isolated a total of 361 epiphytic and 373 endophytic bacteria from leaves, stems and roots of two soybean cultivars. He designed two methodologies for the confirmation of nitrogen fixing bacteria. They were (i) bacterial growth in nitrogen free medium (NFb medium) and (ii) PCR specific for the *nifH* gene (encode nitrogenase protein Componet II). The NFb medium methodology revealed that 60% of analysed endophytic and 69% of epiphytic isolates were able to grow in nitrogen free medium. These isolates belonged to α and β *Proteobacteria*, although the predominant groups were *Enterobacteriaceae* and *Pseudomonadaceae*. The PCR method revealed the presence of *nifH* in 21% of the endophytic isolates, which were identified as *Acinetobacter calcoaceticus*, *Burkholderia* sp., *Pseudomonas* spp., *Ralstonia* sp. and species belonging to the *Enterobacteriaceae* group.

High N_2-fixing activities have been estimated in the high or excessive carbohydrate producing plants, like as sugarcane, pineapples and sweet potato. Rice

endophytic bacteria which fix N_2 belong to the following groups; *Pseudomonas* sp. (You and Zhou, 1989), *Azoarcus* sp. (Hurek *et al*., 1994), *Burkholderia* sp. (Engelhard *et al.*, 2000), *Herbaspirillium seropedicae* (Olivares *et al.*,1996), *Rhizobium leguminosarum* (Yanni *et al.*, 1997), *Serratia* sp. (Sandhiya *et al.*, 2005), *Klebsiella* sp. (Rosenblueth *et al.*, 2004) and *Azorhizobium caulinodans* (Engelhard *et al.*, 2000).

The isolation of diazotrophic endophytes *Pantoea agglomerans* by Asis and Adachi (2003) and *Klebsiella oxytoca* by Adachi *et al.* (2002) apparently support the findings of Yoneyama *et al.* (1998) on the possible contribution of biological nitrogen fixation in Japanese sweet potato cultivars. Yoneyama *et al.* (1998) reported based on natural ^{15}N abundance method, the estimated amount of nitrogen derived through biological nitrogen fixation ranged from 26 to 44% in field grown sweet potato.

2.7. Endophytic colonization of plant:

A group of scientists observed that endophytic *Azocarus* sp. infected plants through the emergence points of lateral roots and root tips via the action of a bacterial endoglucanase (Rheinhold-Hurek *et al*., 2006). In addition, transposon mutants lacking the activity of this endoglucanase colonized rice plants to a significantly lesser extent. This group subsequently showed that deletion mutants of the *pilT* and *pilA* genes in this bacterium abolished bacterial twitching and motility as well as the endophytic colonization of the roots of rice plants (Böhm *et al*., 2007), where *pil*T and *pil*A encode the pilus retraction protein and the pilin structural protein, respectively. In another study of the colonization of host plants by bacterial endophytes, one group showed that the *gumD* gene from the nitrogen-fixing

endophyte *Gluconacetobacter diazotrophicus* which is involved in exopolysaccharide biosynthesis is required for biofilm formation and subsequent plant colonization (Meneses *et al*., 2011). From the same group, it was later demonstrated the importance of endophyte colonization in rice plants of the *gr* and *sod* genes, a glutatione redutase and a superoxide dismutase, analyzed in the same N_2-fixing strain *G. diazotrophicus* (Alquéres *et al*., 2013).

Toumatia *et al.* (2016) established an experiment for colonization of the *Streptomyces mutabilis* strain IA1. It was visualized 10 days post inoculation on the rhizoplane and inside the endorhiza as well as inside tissues of caryopses of plants, using EUB338mix-FLUOS and HGC69a probe coupled with cy5 fluorochrome allowing visualization of the bacterium as fluorescent yellow/orange colored. Some root zones were colonized rarely by strain IA1. Neither root tip colonization nor root emergence site colonization was observed for the *S. mutabilis* strain IA1 on the rhizoplane of the root systems of the wheat seedlings. Root elongation zone was colonized rarely. At the root hair zone level, strain IA1 was however detected as hyphae forms colonizing intensively the surfaces of root hairs. Strain IA1 was additionally detected colonizing root internal tissues such as the exodermis, cortex, endodermis as well as the central cylinder close to xylem vessels. *S. mutabilis* strain IA1 was also visualized inside the caryopses. Short hyphae were visualized particularly inside parts of the dry fruit part of caryopses, e.g. in the epicarp, mesocarp and endocarp cell layers.

Rangel de Souza *et al.* (2015) reported that *Gluconacetobacter diazotrophicus* efficiently promoted *A. thaliana* plant growth at 50 days after inoculation. Inoculated

plants showed higher whole canopy photosynthesis, lower whole plant transpiration, and increased water-use efficiency. The bacterium colonized preferentially root xylem. CFU counting assays revealed 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. In leaves, no bacteria were detected by CFU at 14 and 50 DAI; GD-Kan was only detected at 28 DAI, but at very low concentrations (10^2 CFU/g). At 50 DAI, an increase in number of leaves, leaf area, and shoot and root dry weight was observed. Number of leaves was 28.9±4.06 and 25.9±3.7 for inoculated and non-inoculated plants, respectively. Total leaf area per plant was approximately 5.0 cm^2 higher in inoculated plants than in control plants. Dry weight results confirmed increased leaf and root mass in inoculated plants.

Sandhiya *et al*. (2005) isolated nitrogen fixing endophytic *Serratia* sp. from rice and re-colonization ability of *Serratia* sp. in the rice seedling as endophyte was studied under laboratory condition. For detecting the re-colonization, *Serratia* sp. was marked with reporter genes (egpf and Km^r) using transposon mutagenesis. The conjugants were able to colonize well in the stem portion of rice seedling and very few colonized the leaf and root portion on the 15^{th} day after inoculation. Even though the entry of the endophyte was through root, the colonization was poor in the root region of the rice seedlings. Similarly, the *gus A* marked *Serratia marcescens* was able to colonized endophytically in stem and leaf tissues of rice seedlings (Gyaneshwar *et al*., 2001). Microscopic analysis of ultra microtrome section of the roots inoculated with *Azorhizobium caulinodans* in the presence of naringenin by the organism abundantly in rice seedlings (Gopalaswamy *et al*., 2000).

Elbeltagy *et al*. (2001) designed an experiment to examine whether *Herbaspirillum* sp. strain B501 isolated from wild rice, *Oryza officinalis*, endophytically colonizes the rice plants. The *gfp* gene encoding green fluorescent protein (GFP) was introduced into the bacteria. Observations by fluorescence stereomicroscopy showed that the GFP-tagged bacteria colonized shoots and seeds of aseptically grown seedlings of the original wild rice after inoculation of the seeds. Conversely, for cultivated rice *Oryza sativa*, no GFP fluorescence was observed for shoots and only weak signals were observed for seeds. Observations by fluorescence and electron microscopy revealed that *Herbaspirillum* sp. strain B501 colonized mainly intercellular spaces in the leaves of wild rice. Colony counts of surface-sterilized rice seedlings inoculated with the GFP-tagged bacteria indicated significantly more bacterial populations inside the original wild rice than in cultivated rice varieties.